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(43) Title: TISSUE-SPECIFIC TRANSCRIPTION OF DNA SEQUENCE ENCODING A HETEROLOGOUS ENZYME FOR USE IN PRODRUG THERAPY TO LUNG CANCER			

## (57) Abstract

The invention relates to the use of a molecular chimera with a prodrug in the therapy of lung cancer, the molecular chimera comprising a transcriptional regulatory DNA sequence derived from a gene encoding a lung-associated protein or a neuroendocrine marker protein and, operatively linked to the transcriptional regulatory DNA sequence, DNA sequence encoding a heterologous enzyme capable of catalysing the conversion of the prodrug into an agent toxic to a lung cancer cell. The use of lung specific promoters in a GDEPT or VDEPT approach allows specific targeting of lung cancer cells.

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TISSUE-SPECIFIC TRANSCRIPTION OF DNA SEQUENCE ENCODING A HETEROLOGOUS  
ENZYME FOR USE IN PRODRUG THERAPY TO LUNG CANCER

The present invention relates to enzyme prodrug therapy and, in particular, to the application of this form of therapy to lung cancer.

Lung cancer is a major type of cancer in the countries of Western Europe and North America. For example, it is the most common lethal cancer in the United States. It was estimated that 172,000 people would develop lung cancer in the U.S. in 1994 and about 153,000 people would die of it. The number of deaths from lung cancer is steadily increasing. The prevalence of lung cancer in developing countries is relatively low but is expected to increase sharply with the fast spreading of tobacco smoking. It is estimated that by the year 2000, deaths related to lung cancer will increase worldwide to about 2 million, mainly as a result of an increase in cigarette smoking by young adults.

Multiple categories of cancer commonly arise in the lung. Clinically, and therapeutically, about 25% of lung cancer cases are classified as small cell lung carcinoma (SCLC). The other 75% of cases consist of squamous carcinomas, large cell carcinomas, and adenocarcinomas, and are referred to collectively as non-small cell lung carcinoma (NSCLC).

For SCLC, combination chemotherapy forms the cornerstone of therapy. Because of its relatively rapid growth rate, and its tendency to metastasize, SCLC can rarely be treated surgically. In the absence of treatment, median survival in SCLC is only a few months. After aggressive and toxic combination chemotherapy, only 10% of patients with SCLC will be alive at 2 years after diagnosis and 5% at 5 years.

For NSCLC, surgery is the major curative modality for patients without demonstrable metastatic disease. However, only a minority of cases can be cured by surgical resection. Chemotherapy and chest radiotherapy are also seldom curative. The overall 5-year survival for newly diagnosed cases of NSCLC is only 10% to 15%.

There has been no improvement in the survival rate for lung cancer patients in decades, which is a reflection both of the lack of a satisfactory screening test that could detect it in the early stages and, up to the present time, of the lack of truly

effective treatment with clear survival benefits. With the adoption in the 1970s of combination chemotherapy for SCLC, and novel surgical approaches for NSCLC, median survival improved slightly. However, very little further improvements have occurred since. For example, the 5-year survival of Americans afflicted with lung cancer improved only slightly from 12% to 13%, from 1974 through 1987 (Ries *et al.* 1991 NIH Publication 91-2789).

A therapeutic plateau has now been reached, and it is clear that new approaches are needed for lung cancer.

Gene or virus directed enzyme prodrug therapy (GDEPT or VDEPT) is potentially less toxic and more efficient as a therapy for cancer than existing therapies. GDEPT or VDEPT involves the use of a gene encoding an enzyme that is capable of converting a relatively nontoxic prodrug to its active, e.g. cytotoxic, form.

WO-A-90 07936 proposes a treatment for an infection or a hyperproliferative disorder which is characterised by the presence, in the affected cells, of a trans-acting factor capable of regulating gene expression by inserting into the cells a polynucleotide construct having a cis-acting regulatory sequence which is regulated by the trans-acting factor and an effector gene which renders said cell susceptible to protection or destruction. For example, the cis-acting region may be homologous to the HIV tar region, and the effector gene may encode ricin A or HSV-1 thymidine kinase. Upon infection with HIV, the HIV tat protein activates the tar region, and induces transcription and expression of ricin A, resulting in cell death, or of HSV-1 tk, resulting in cell death upon treatment with dideoxynucleoside agents such as acyclovir and gancyclovir.

EP-A-0 334 301 describes methods for the delivery of vectors using recombinant retrovirus wherein the vector construct directs the expression of a protein that activates a compound with little or no cytotoxicity into a toxic product in the presence of a pathogenic agent, thereby effecting localised therapy to the pathogenic agent.

EP-A-0 415 731 describes molecular chimaeras for use with prodrugs, comprising transcriptional regulatory DNA sequences capable of being selectively activated in a mammalian cell, and a DNA sequence operatively linked to the transcriptional

regulatory DNA sequence and encoding a heterologous enzyme capable of catalysing the conversion of the prodrug into an agent toxic to the cell. Transcriptional regulatory sequences specifically mentioned are albumin, alfafetoprotein, carcino-embryonic antigen, tyrosine hydroxylase, choline acetyl transferase, neuron-specific enolase, glial  
5 fibro acidic protein, insulin, gamma glutamyl/transpeptidase, dopa decarboxylase, HER2/neu and N-myc oncogenes. Specific prodrug/enzyme combinations disclosed are purine or pyrimidine analogs/VZV tk, FC/cytosine deaminase, phenoxyacetamide derivatives of adriamycin and melphalen/penicillin V amidase, phosphate salt of etoposide, adriamycin or mitomycin C /alkaline phosphatase, para-N-bis-(2-Cl-ethyl)  
10 aminobenzylglutamic acid/carboxypeptidase G2.

According to one aspect, the present invention provides the use of a molecular chimaera for the manufacture of a medicament for use with a prodrug in the therapy of lung cancer, the molecular chimaera comprising a transcriptional regulatory DNA  
15 sequence derived from a gene encoding a lung-associated protein or a neuroendocrine marker protein and, operatively linked to the transcriptional regulatory DNA sequence, DNA sequence encoding a heterologous enzyme capable of catalysing the conversion of the prodrug into an agent toxic to a lung cancer cell.

According to another aspect the present invention provides a molecular chimaera for use in therapy of lung cancer with a prodrug, the molecular chimaera comprising a transcriptional regulatory DNA sequence derived from a gene encoding a lung-associated protein or a neuroendocrine marker protein and, operatively linked to the transcriptional regulatory DNA sequence, DNA sequence encoding a heterologous  
25 enzyme capable of catalysing the conversion of the prodrug into an agent toxic to a lung cancer cell.

The molecular chimaera of the present invention may be made utilising standard recombinant DNA techniques.

30 Lung is not the site for dose limiting toxicity of most anticancer agents because airway epithelial cells are well differentiated and non-dividing. Therefore, lung specific activation of prodrugs improves the selectivity of these agents. Also, the promoter elements for lung-specific genes can be used to target selectively the lung metastatic  
35 disease localized in other tissues.

The majority of small cell lung carcinomas and about 30% of non-small cell lung cancer are of neuroendocrine origin. Neuroendocrine (NE) tumors usually produce multiple markers for NE differentiation such as creatine kinase, neuron-specific enolase, L-dopa decarboxylase, chromogranin A, neural cell adhesion molecule, Leu-7, gastrin releasing peptide, synaptophysin, calcitonin, serotonin insulinoma-associated peptide and ACTH (a hormone produced from a precursor protein called proopiomelanocortin (POMC)). These peptide and amine products are used to identify the specific tumor types. NE tumor cells selectively express the genes for most of these markers because the transcriptional regulatory sequence (TRS) elements of these genes are functional only in NE tumors and in a small nest of neurons, endocrine and ganglion cells of the central and peripheral nervous systems. Therefore, the TRS elements for NE marker genes are highly specific for cancers of neuroendocrine origin.

TRS elements have been isolated and characterised for a number of NE marker genes.

The proopiomelanocortin (POMC) gene codes for the precursor of multiple peptide hormones, including ACTH, and is normally expressed only in the anterior pituitary. The sequence of the human POMC gene, including 680 base pairs preceeding the transcriptional initiation site, have been determined (Takahashi *et al.*, Nucl. Acids Res., 11, 6847-6858 (1983)).

The chromogranin A (CgA) gene codes for an acidic glycoprotein which is involved in hormone packaging and secretion in neuroendocrine cells. The sequence of the human CgA gene including 250 base pairs preceeding the transcriptional initiation site have been determined (Mouland *et al.*, J. Biol. Chem., 269, 6918-6926 (1994)).

Gastrin-releasing peptide (GRP) is a 27-amino acid peptide hormone. Although found in neurons in the gastrointestinal tract and in the brain, highest levels of GRP are found in fetal lung and SCLC (Moody *et al.*, Science, 214, 1246-1248 (1981)). Many small cell lung cancers overexpress GRP as well as the GRP receptor, and in these cells binding of GRP to its receptor may act as an autocrine mitogenic stimulus (Cuttitta *et al.*, Nature, 316, 823-826 (1985)). A functional analysis of the 5-prime

flanking region of the human GRP gene has been carried out which defined a DNA fragment which conferred SCLC-specific expression to a heterologous reporter gene (Nagalla and Spindel, *Cancer Res.*, **54**, 4461-4467 (1994)).

- 5 Proteins shown to be lung associated or lung-specific include uteroglobin or Clara cell 10 kD protein (CC10), which is a marker for lung and a marker for endometrial differentiation. It is the predominant secreted protein of lung Clara cells which line the bronchiolar epithelium. The protein is a homodimer with a molecular weight of 17 kD. In humans, detectable levels of uteroglobin is also expressed in trachea and prostate.
- 10 The physiological role of the protein is not completely understood but is known to possess anti-inflammatory activity and to inhibit phospholipase A<sub>2</sub>.

- Other such proteins include pulmonary surfactant which is composed of a mixture of lipids and surfactant proteins specifically expressed in the respiratory epithelium. Their
- 15 main function involves reduction in surface tension in the alveolar space and hence prevention of alveolar collapse. There are four surfactant proteins, A, B, C, and D each interacting with the lipid component differently (Weaver and Whitsett, *Biochem. J.*, **273**, 249-264 (1991)). Of these, the regulatory element for SP-B, which direct lung specific transcription, has been identified as a 259 bp fragment (Bohinski *et al.*, *J. Biol. Chem.*, **268**, 11160-11166 (1993) and Tami *et al.*, *DNA*, **8**, 75-86 (1989)).
- 20

- According to the present invention, specificity of expression of the heterologous enzyme for lung cancer cells and hence selective conversion of the prodrug to the active cytotoxic form is achieved by the use of the TRS derived from a gene encoding
- 25 a lung-associated or NE-marker protein. A number of specific enzyme prodrug combinations can be used in association with this lung specific TRS.

- The term "heterologous enzyme" as used herein means any enzyme not present naturally in the targetted lung cancer cell. This comprises non-mammalian enzymes
- 30 such as those derived from yeast or bacteria and mammalian enzymes including naturally occurring mutant mammalian enzymes or mutant mammalian enzymes which have been generated being recombinant DNA technology.

- Suitable enzymes for use according to the present invention include any having a
- 35 catalytic activity appropriate to the conversion of a prodrug to a therapeutically active

compound. Such enzymes include cytosine deaminase which converts the prodrug 5-fluorocytosine to toxic 5-fluorouracil, human carboxypeptidase A1 which converts the prodrug para-N-bis(2-chloroethyl)-aminobenzoyl glutamic acid into benzoic acid mustard, the enzyme alkaline phosphatase which converts the prodrugs etoposidephosphate, doxorubicin phosphate and mitomycin phosphate into the corresponding toxic dephosphorylated metabolite and the enzyme penicillin-B-amidase which converts a prodrug which is a phenylacetamide derivative of doxorubicin or melphalan into its corresponding toxic metabolite.

Another preferred enzyme for use according to the present invention is  $\beta$ -lactamase which has particular advantages in terms of the range of toxic agents which can be presented in the form of prodrugs capable of conversion to the active agent by means of the enzyme. In principle any toxic agent can be converted to such a prodrug by conjugation with another compound through a bond capable of being cleaved by  $\beta$ -lactamase. According to one particularly advantageous embodiment, conjugates are formed between the toxic agent and a cephalosporin. Specific examples include conjugates of 5-fluorouracil, methotrexate and adriamycin linked in each case to a cephalosporin (see WO-A-94 01 137 and EP-A-0 382 411) and cephalosporin mustards (see EP-A-0 484 870). In each case the cephalosporin/toxic agent conjugate shows markedly reduced toxicity but can be converted to the active form by  $\beta$ -lactamase thus making it suitable for use as a prodrug in GDEPT. Other toxic agents can be linked to cephalosporins in a similar way.

Prodrugs for use according to the present invention may thus be based on any compound showing a suitable chemotherapeutic effect. Preferred cytotoxic compounds include nitrogen mustard agents, antifolates, nucleoside analogs, the vinca alkaloids, the anthracyclines, the mitomycins, the bleomycins, the cytotoxic nucleosides, the pteridine family of drugs, the podophytophyllotoxins, the sulfonylureas (as described in EP-A-0 222,475) and low-molecular-weight toxins such as the trichothecenes and the colchicines. Particularly examples include doxorubicin, daunorubicin, aminopterin, methotrexate, taxol, methopterin, dichloromethotrexate, mitomycin C, porfirimycin, 5-fluorouracil, 6-mercaptopurine, cytosine arabinoside, podophyllotoxin, etoposide, melphalan, vinblastine, vincristine, desacetylvinblastine hydrazide, leurosine, vindesine, leurosine, trichothecene and desacetylcolchicine.



According to the present invention, the molecular chimaera is selectively expressed in a target lung cancer cell population. This may be taken to mean that the chimaera is expressed at a higher level in the target than in the non-target cell population and is preferably expressed predominantly or exclusively in that population. Selective expression is achieved by inclusion of the target-cell specific TRS (promoter with or without enhancer) as described above but may also be enhanced by the method of delivery of the chimaera to the target cell. Methods capable of providing target cell specific delivery of the chimaera, with subsequent stable integration and expression, include the techniques of calcium phosphate transfection, electroporation, microinjection, liposomal transfer, ballistic barrage or retroviral infection or infection using adenovirus or adeno-associated virus. For a review of this subject see Biotechniques 6(7) (1988).

Selectivity may be obtained by a variety of such techniques. Physiologically localised delivery of the chimaera for the target cells will reduce the possibility of non-target cells expressing the chimaera. This may be achieved when for example using retroviral or liposome mediated delivery and would involve direct injection to a blood vessel known to supply the target cells. Selectivity may also be obtained using retroviral mediated chimaera delivery in the therapy of hyperproliferative disorders. Retroviruses only infect dividing cells and would therefore only introduce chimaeras to dividing cells. Liposome technology permits the delivery of the chimaera contained therein to be targeted to a particular cell type based on appropriate modifications made to the liposome coat structure

The technique of retroviral infection of cells to integrate artificial genes employs retroviral shuttle vectors which are known in the art (see for example Mol. and Cell Biol. 6, 2895-2902 (1986)). Essentially retroviral shuttle vectors are generated using the DNA form of the retrovirus contained in a plasmid. These plasmids also contain sequences necessary for selection and growth in bacteria. Retroviral shuttle vectors are constructed using standard molecular biology techniques well known in the art. Retroviral shuttle vectors have the parental endogenous retroviral genes (e.g. gag, pol and env) removed and the DNA sequence of interest inserted, such as the molecular chimaeras which have been described. They however contain appropriate retroviral regulatory sequences for viral encapsidation, proviral insertion into the target genome, message splicing, termination and polyadenylation. Retroviral shuttle vectors have

been derived from the Moloney murine leukaemia virus (Mo-MLV) but it will be appreciated that other retroviruses can be used such as the closely related Moloney murine sarcoma virus. Certain DNA viruses may also prove to be useful as a delivery system. The bovine papilloma virus (BPV) replicates extrachromosomally so that delivery system based on BPV have the advantage that the delivered gene is maintained in a nonintegrated manner. Adenoviruses and adeno-associated viruses may also be used.

Thus according to a further aspect of the present invention there is provided a retroviral shuttle vector containing a molecular chimaera as hereinbefore defined.

The advantages of a retroviral-mediated gene transfer system are the high efficiency of the gene delivery to the targeted tissue, sequence specific integration regarding the viral genome (at the 5' and 3' long terminal repeat (LTR) sequences) and little rearrangements of delivered DNA compared to other DNA delivery systems.

Accordingly in a preferred embodiment of the present invention there is provided a retroviral shuttle vector comprising a DNA sequence comprising a 5' viral LTR sequence, a cis acting psi encapsidation sequence, a molecular chimaera as hereinbefore defined and a 3' viral LTR sequence.

In a preferred embodiment and to help eliminate non-target-specific expression of the molecular chimaera, the molecular chimaera is placed in opposite transcriptional orientation to the 5' retroviral LTR. In addition a dominant selectable marker gene may also be included which is transcriptionally driven from the 5' LTR sequence. Such a dominant selectable marker gene may be the bacterial neomycin-resistance gene NEO (aminoglycoside-3-phosphotransferase type II) which confers on eukaryotic cells resistance to the neomycin analogue G418 sulphate (Geneticin - trade mark). The NEO gene aids in the selection of packaging cells which contain these sequences.

The retroviral vector used may be based on the Moloney murine leukaemia virus but it will be appreciated that other vectors may be used. Such vectors containing a NEO gene as a selectable marker have been described, for example, the N2 vector (Science, 230, 1395-1398 (1985)).

A theoretical problem associated with retroviral shuttle vectors is the potential of retroviral long terminal repeat (LTR) regulatory sequences transcriptionally activating a cellular oncogene at the site of integration in the host genome. This problem may be diminished by creating SIN vectors. SIN vectors are self-inactivating vectors which contain a deletion comprising the promoter and enhancer regions in the retroviral LTR.

The LTR sequences of SIN vectors do not transcriptionally activate 5 or 3 genomic sequences. The transcriptional inactivation of the viral LTR sequences diminishes insertional activation of adjacent target cell DNA sequences and also aids in the selected expression of the delivered molecular chimera SIN vectors are created by removal of approximately 299 bp in the 3 viral LTR sequence (Biotechniques, 4, 504-512 (1986)). Thus preferably the retroviral shuttle vector of the present invention are SIN vectors.

Since the parental retroviral gag pol and env genes have been removed from these shuttle vectors a helper virus system may be utilised to provide the gag pol and env retroviral gene products trans to package or encapsidate the retroviral vector into an infective virion. This is accomplished by utilising specialised "packaging" cell lines which are capable of generating infectious synthetic virus yet are deficient in the ability to produce any detectable wild-type virus. In this way the artificial synthetic virus contains a chimera of the present invention packaged into synthetic artificial infectious virions free of wild-type helper virus. This is based on the fact that the helper virus that is stably integrated into the packaging cell contains the viral structural genes but is lacking the psi site and cis acting regulatory sequence which must be contained in the viral genomic RNA molecule for it to be encapsidated into an infectious viral particle.

Accordingly the present invention provides an infective virion comprising a retroviral shuttle vector as hereinbefore described said vector being encapsidated within viral proteins to create an artificial infective replication-defective retrovirus.

In addition to removal of the psi site additional alterations can be made to the helper virus LTR regulatory sequences to ensure that the helper virus is not packaged in virions and is blocked at the level of reverse transcription and viral integration.

Alternatively helper virus structural genes (i.e. gag pol and env) may be individually and independently transferred into the packaging cell line. Since these viral structural genes are separated within the genome of the packaging cell, there is little chance of covert recombinations generating wild-type virus.

In a further aspect of the present invention there is provided a method for producing infective virions of the present invention by delivering the artificial retroviral shuttle vector comprising a molecular chimaera of the invention as hereinbefore described into a packaging cell line.

The packaging cell line may have stably integrated within it a helper virus lacking a psi site and other regulatory sequence as hereinbefore described or alternatively the packaging cell line may be engineered so as to contain helper virus structural genes within its genome.

The present invention further provides an infective virion as hereinbefore described for use in therapy particularly for use in the treatment of lung cancer.

The infective virion according to the invention may be formulated by techniques well known in the art and may be presented as a formulation with a pharmaceutically acceptable carrier therefor. Pharmaceutical acceptable carriers in this instance may comprise a liquid medium suitable for use as vehicles to introduce the infective virion into the patient. An example of such a carrier is saline. The infective virion may be a solution or suspension in such a vehicle. Stabilisers and antioxidants and or other excipients may also be present in such pharmaceutical formulations which may be administered to a mammal by any conventional method e.g. oral or parenteral routes. In particular the infective virion may be administered by intra-venous or intra-arterial infusion.

Accordingly the invention also provides pharmaceutical formulations comprising a molecular chimaera of the present invention contained within one of, an infective virion or a liposome or a packaging cell mix, in admixture with a pharmaceutically acceptable carrier, and pharmaceutical formulations comprising a molecular chimaera virion, vector, liposome or packaging cell mix of the present invention in admixture with a pharmaceutically acceptable carrier.

Additionally the present invention provides methods of making pharmaceutical formulations as herein described comprising mixing an artificial infective virion containing a molecular chimaera with a pharmaceutically acceptable carrier.

The invention also includes the use of any molecular chimaera, vector, virion, liposome or pharmaceutical formulation of the present invention in human therapy and in the manufacture of a medicament for use in the treatment of pathological states.

The invention also includes methods of medical therapy comprising the use of any molecular chimaera, vector, virion, liposome or pharmaceutical formulation of the present invention.

Also included within the scope of the present invention is a protein encoded by a molecular chimaera of the present invention and any combination of such a protein and a prodrug which can be catalysed by the enzyme component of that protein.

The precise dosage to be administered to a patient will ultimately be dependent upon the discretion and professional judgement of the attendant physician and will be a product of the particular targetting mechanism chosen. References contained herein to the efficiency of targetting of retroviruses, liposome etc. may be used to determine appropriate dosage levels.

The amounts and precise regime in treating a mammal, will of course depend on a number of factors including the type and severity of the condition to be treated. However, for carcinoma an arterial or intravenous infusion of the artificial infective virion at a titre of between  $2 \times 10^5$  and  $2 \times 10^7$ , for example  $5 \times 10^5$ ,  $8 \times 10^5$ ,  $2 \times 10^6$ ,  $5 \times 10^6$  or  $8 \times 10^6$ , colony forming units per ml (CFU/ml) infective virions is likely to be suitable for a typical tumor. Total amount of virions infused will be dependent on tumour size and would probably be given in divided doses.

The dose of prodrug will advantageously be in the range of 0.1 to 250mg per kilogram body weight of recipient per day, preferably 0.1 to 100mg per kilogram bodyweight.

The invention is illustrated further in the following examples in which reference is made to the accompanying drawings in which:

FIG 1 shows the results of use of the POMC promoter relative to the CMV promoter in small cell lung tumours.

FIG 2 shows the results of use of the CgA promoter relative to the CMV promoter in lung tumours.

FIG 3 shows the results of expression of the human uteroglobin promoter in different human tumor lines.

FIG 4 shows expression of the surfactant protein-B promoter in different human tumour lines.

FIG 5 shows cellular location of  $\beta$ -lactamase activity in mammalian cells transfected with  $\beta$ -lactamses constructs.

#### EXAMPLE 1

##### Cloning of the POMC promoter

A 785 base pair sequence was amplified via PCR from human fibroblast genomic DNA (Clontech, Palo Alto, CA) using the following two primers: JM30;

5'-TGACAATCGCGACTGCTCTTCACAGCATCACCTCTCCC (39-mer; SEQ ID NO 1)

and JM31;

5'-GGATCCCGGGGAAAGAGCACGGGTCC (26-mer SEQ ID NO 2).

JM30 represents POMC sequences ending at -680 of the sequence defined by Takahashi *et al.* (*supra*) with flanking sequence containing a Nru I restriction site.

JM31 represents POMC sequences ending at +105 in the region containing the 5-prime untranslated portion of the POMC mRNA. The PCR reaction was carried out for 25 cycles using standard conditions and using Vent polymerase (New England Biolabs, Inc.). PCR thermal cycling conditions were 95°C, 1 min; 65°C, 3 min; 70°C, 2 min; 92°C, 1 min; 72°C, 2 min; 25 cycles then 75°C, 10 min. This PCR product was gel-purified using the Glass-Max kit (Life Technologies, Inc.) and subsequently used

for a second PCR reaction using JM30 in combination with an internal primer. The second primer consisted of the following sequence: JM32:  
5'-TGACAAAAGCTTCGGCCTCTCTCGGTCGCGGCTCTTC (37-mer; SEQ ID NO 3).  
JM32 represents POMC sequences ending at +22 in the region representing the 5'-untranslated portion of the POMC mRNA. PCR was carried out as above except that thermal cycling conditions were 95°C, 1 min; 92°C, 1 min; 70°C, 1 min; 72°C, 2 mins; 25 cycles then 75°C; 10 min. This PCR product was gel-purified using the Glass-Max kit (Life Technologies, Inc.), restriction digested with Nru I and Hind III (Life Technologies, Inc.) and ligated to pRc-CMV (Invitrogen, Inc) containing the secretory  $\beta$ -lactamase coding region (see Example 8 below). The sequence of the promoter was confirmed using the dideoxy chain-terminator sequencing method (Sanger *et al.*, 1977).

## EXAMPLE 2

### Cloning of the Chromogranin A Promoter

250 base pairs of sequence preceeding the transcription initiation site of the CgA promoter have been cloned. To clone the CgA promoter itself, the sequence was amplified from a human fibroblast genomic DNA preparation (Clontech, Palo Alto, CA, USA). The forward oligo used was CGN3:

5'-TGACAAATCGCGACTCTTGGAACACAGATACCCGTCGC (37-mer; SEQ ID NO 4), which contains a flanking Nru I site (TCGCGA) and sequences beginning at -211 of the human chromogranin A promoter. The reverse primer was CGN4:

5'-TGACAAAAGCTTCTCGAGCACTGCAGTGGCAGGAGC (36-mer; SEQ ID NO 5) which consisted of a flanking Hind III site and sequences beginning at +37 of the 5'-untranslated region of the chromogranin A promoter. The PCR conditions were essentially the same as were used for the POMC promoter (see Example 1 above).

## EXAMPLE 3

### Proopiomelanocortin Promoter

The sequence of the human POMC gene, including 680 base pairs preceeding the transcriptional initiation site, have been determined (Takahashi *et al.*, *supra*). The 680 base pair control region of the POMC promoter was fused to the  $\beta$ -lactamase coding region in order to utilize  $\beta$ -lactamase as a reporter of promoter strength.

Transient transfections were used to evaluate the expression of different promoters. Transfections were carried out by liposome-mediated DNA delivery using lipofectamine (Life Technologies, Inc., Gaithersburg, MD, USA). Experiments were performed according to manufacturer's instructions, varying the number of cells, amount of transfection reagent, and amount of DNA to determine optimum conditions. Typically, 60 x 15mm tissue culture plates containing approximately  $3 \times 10^5$  to  $1 \times 10^6$  cells were employed.

The POMC- $\beta$ -lactamase construct was transfected into seven cell lines (Figure 1). The relative strength of the promoter was quantitated by comparing the magnitude of expression of the POMC- $\beta$ -lactamase construct to expression observed in parallel transfections using a CMV promoter- $\beta$ -lactamase construct. The POMC promoter displayed apparent selectivity towards small cell carcinoma lines.

#### EXAMPLE 4

##### The chromogranin A promoter

The sequence of the human CgA gene including 250 base pairs preceding the transcriptional initiation site have been determined (Mouland *et al.*, *supra*). The strength and specificity of the promoter were evaluated as described for POMC, using the  $\beta$ -lactamase gene as a reporter. This promoter was found to be active in all lung lines tested (Figure 2). Expression of CgA promoter was 0.4% of CMV in a colon line (WiDr) and was 1% of CMV in an ileocecal (HCT-8) line.

#### EXAMPLE 5

##### The Gastrin-Releasing Peptide Promoter

Many small cell lung cancers overexpress GRP as well as the GRP receptor, and in these cells binding of GRP to its receptor may act as an autocrine mitogenic stimulus (Cuttitta *et al.*, *Nature*, 316, 823-826 (1985)). Recently, a functional analysis of the 5-prime flanking region of the human GRP gene has been carried out (Nagalla and Spindel, *Cancer Research*, 54, 4461-4467 (1994)). A DNA fragment was defined which conferred SCLC-specific expression to a heterologous reporter gene. This sequence may be used to regulate expression of an activating enzyme gene.

#### EXAMPLE 6

##### The Uteroglobin Promoter



A 465 base pair element of the 5'-flanking region of the human uteroglobin promoter was isolated. The element was placed in front of a  $\beta$ -lactamase reporter gene so that the reporter was under the transcriptional control of the uteroglobin promoter. Transient transfections using lipofectamine were carried out using the CMV promoter as a control as described above. Data obtained so far (Figure 3) suggests that expression of the uteroglobin promoter is substantially restricted to non-small cell lung cancer lines.

#### EXAMPLE 7

##### Surfactant Protein B Promoter

Pulmonary surfactant is composed of a mixture of lipids and surfactant proteins. These surfactant proteins are specifically expressed in the respiratory epithelium. Their main function involves reduction in surface tension in the alveolar space and hence prevent alveolar collapse. There are four surfactant proteins, A, B, C, and D each interacting with the lipid component differently (Weaver and Whitsett, *supra*). Of these, the regulatory element for SP-B, which direct lung specific transcription, has been identified as a 259 bp fragment (Bohinski *et al.*, and Tami *et al.*, *supra*). This promoter sequence was generated using overlapping oligonucleotides in a PCR based strategy. The promoter element was tested for directing transcription of a  $\beta$ -lactamase reporter, *in vitro*, in various tumor lines and the results are shown in Figure 4.

#### EXAMPLE 8

##### (i) Cloning of *E. coli* $\beta$ -Lactamase for Human Cell Expression

We have constructed unique DNA constructs containing the bacterial  $\beta$ -lactamase gene which, when delivered to human cells, result in expression of functional  $\beta$ -lactamase. The advantages of  $\beta$ -lactamase as a prodrug activating enzyme are 1) the enzyme is kinetically very efficient and 2) because of a unique activation mechanism, a prodrug of virtually any drug can be made as an efficient substrate for the enzyme. The implications of this to cancer therapy is that it permits the use of combination prodrug therapy to counter resistance phenomena as well as allows one to choose drugs appropriate to the tumor target. To target lung cancer, prodrugs of methotrexate (5798W93) and 5-fluorouracil (1614W94) have been synthesized.  $\beta$ -lactamase constructs have been created which give rise to secreted, intracellular and membrane-anchored forms.

### (iii) Construction of Secretory $\beta$ -lactamase Constructs

To create a DNA construct which would express secretory  $\beta$ -lactamase in human cells, the coding region of TEM  $\beta$ -lactamase (Sykes and Matthews, J. Antimicrob. Chemo., 2, 115-157 (1976); Ambler and Scott, Proc. Natl. Acad. Sci. USA, 75, 3732-3736 (1978)) was used. Since it exists in the periplasm of bacteria, the unmodified coding region of TEM  $\beta$ -lactamase contains a signal peptide (Sutcliffe, Proc. Natl. Acad. Sci. USA, 75, 3737-3741 (1978)). Sequences useful for the cloning and expression of this gene in a eukaryote were added to flanking sequence during PCR by including the sequences in the PCR primers. The sequence of the forward primer JM1 was:

5'-TTGCATAAGCTTGCCACCATGAGTATTCAACATTTCCGTGTC (42-mer; SEQ ID NO 6). The sequence of the reverse primer JM2 was:

5'-GATCTGTCTAGATTACCAATGCTTAATCAGTGAGGC (36-mer; SEQ ID NO 7). The forward primer contains a Hind III restriction site (AAGCTT) for subsequent cloning of the PCR product, and a sequence (GCCACC) which confers optimal translation efficiency in vertebrates (Kozak, J. Cell Biol. 115, 887-903 (1991)) immediately 5-prime to the initiator methionine codon (ATG) of the  $\beta$ -lactamase coding region. The reverse primer contains an Xba I restriction site (TCTAGA) adjacent to the stop codon (TAA) of the  $\beta$ -lactamase coding region.

The PCR reaction was carried out for 25 cycles using standard conditions and using Vent DNA Polymerase (New England Biolabs, Inc., Beverly, MA, USA) in 4 mM  $MgSO_4$  and 200  $\mu M$  of each dNTP and 1 pmol/ $\mu l$  forward and reverse primers. PCR thermal cycling conditions were 95°C, 1 min; 60°C, 1 min; 75°C, 1 min, 25 cycles then 75°C, 5 min. The approximately 800 base pair PCR product was gel-purified using the Glass-Max kit (Life Technologies, Inc., Gaithersburg, MD, USA). The purified PCR product was restriction digested with Hind III and Xba I, re-purified by gel electrophoresis, and ligated into the multiple cloning site of the pRc/CMV vector (Invitrogen, Inc., San Diego, CA, USA). The orientation of the  $\beta$ -lactamase insert in this vector places the  $\beta$ -lactamase gene under the transcriptional regulation of the intermediate/early CMV promoter as well as followed a bovine growth hormone poly(A) addition signal. The sequence of the construct (designated pCMV-BL) is shown in SEQ ID NO 8 along with the amino acid sequence of inserted secretory  $\beta$ -lactamase.

### (iii) Construction of Intracellular $\beta$ -lactamase Constructs

To create a DNA construct for expression of intracellular  $\beta$ -lactamase in human, modifications to the terminus of the  $\beta$ -lactamase gene in pCMV-BL were carried out using PCR. The forward primer (JM30) for these reactions consisted of the sequence:

5'-TTGCATAAGCTTGCCACCATGCACCCAGAAACGCTGGTG (39-mer; SEQ ID NO 9).

This forward primer consists of a Hind III restriction site (AAGCTT), a consensus site for optimal translation efficiency (GCCACC) in vertebrates (Kozak, 1991 *supra*) and an ATG initiator codon immediately adjacent to the sequence representing the mature amino-terminus of TEM  $\beta$ -lactamase (Sutcliffe, 1978 *supra*). When used in a PCR reaction in combination with the JM2 reverse primer described above, the resulting PCR product would contain a deleted signal peptide and a new initiator methionine codon adjacent to the mature coding region of  $\beta$ -lactamase. This PCR reaction was carried out using PCR conditions identical to those described for pCMV-BL, except that JM30 was substituted for JM1.

The approximately 700 base pair PCR product was gel-purified using the Glass-Max kit (Life Technologies, Inc., Gaithersburg, MD, USA). The purified PCR product was restriction digested with Hind III and Xba I, repurified by gel electrophoresis, and ligated into the multiple cloning site of the pRc/CMV vector (InVirogen, Inc., San Diego, CA, USA) as described above for pCMV-BL. The sequence of the construct (designated pCMV- $\Delta$ BL) is shown in SEQ ID NO 10 along with the amino acid sequence of inserted intracellular  $\beta$ -lactamase.

### (iv) Construction of Membrane-Bound $\beta$ -lactamase Constructs

A membrane-bound form of  $\beta$ -lactamase would be useful in prodrug therapies since the enzyme is active and does not diffuse from the site of expression and since the external activation of prodrug guarantees bystander effects of the activated drug. This chimeric enzyme may also have potential as a potent immunostimulatory molecule since the membrane location of the protein may enhance its presentation on MHC Class II molecules.

To create a DNA construct which would express  $\beta$ -lactamase inserted in the external portion of the cell membrane in human, a membrane-spanning domain was appended to the carboxy-terminus of the secretory  $\beta$ -lactamase coding region contained in pCMV-BL. The membrane sequence was derived from the human C mu IgM heavy

chain gene (Dorai, Nucl. Acids Res., 17, 6412 (1989)). This was done by fusing a 300 base pair sequence representing the human IgM membrane-spanning domain (from plasmid IgM/TMP/PCRII which contains exons M1 and M2 separated by a single intervening sequence) in-frame to the carboxy-terminus of the secretory  $\beta$ -lactamase gene.

The first step in this process was to delete the termination codon in the  $\beta$ -lactamase sequence contained in pCMV-BL. This was done by PCR amplification of the insert using the forward primer JM1 (see above) in combination with the reverse primer MEM1. MEM1 consists of the sequence:

5'-TGACAATCTAGAGAGGGGGAGGTGAGCGCCGACGAG (36-mer; SEQ ID NO 11).

MEM1 contains sequence representing the carboxy-terminus of secretory  $\beta$ -lactamase excepting the translation termination signal (TAA) which is replaced by an Xba I restriction site. The hexameric Xba I sequence is in-frame with the coding region of  $\beta$ -lactamase and represents a Ser-Arg amino acid sequence. This PCR product was amplified as described above, gel-purified, and cloned into the Hind III and Xba I sites of pRc-CMV. This plasmid was designated pCMV-MEM1.

To attach a carboxy-terminal membrane spanning domain, a 300 base-pair sequence from plgM/TMP/PCRII was amplified with oligos MEM2 and MEM3. MEM2 consists of the sequence:

5'-TGACAATCTAGAGAGGGGGAGGTGAGCGCCGACGAG (36-mer; SEQ ID NO 12).

MEM3 consists of the sequence:

5'-TGACAAGGGCCCTCTGGTCTCCGATGTTCTTC (33-mer; SEQ ID NO 13).

MEM2 represents the amino-terminus of the IgM trans-membrane domain (beginning at nucleotide 489; GenBank Accession #X14939) flanked by an Xba I restriction site (TCTAGA). MEM3 represents the carboxy-terminus of the trans-membrane domain (ending at nucleotide 815; GenBank Accession #X14939) flanked by an Apa I restriction site (GGGCCC). These oligos were used to carry out PCR as described above and the approximately 300 base-pair product was restriction digested, gel-purified, and cloned into the Xba I and Apa I sites of pCMV-MEM1. The sequence of the construct (designated pCMV-BLIgM) along with the amino acid sequence of inserted membrane-anchored  $\beta$ -lactamase is shown in SEQ ID NO 14.

(v) **Determination of Cellular Locations of Targeted  $\beta$ -Lactamase Protein**

Confirmation of the predicted locations of each of the  $\beta$ -lactamase constructs was carried out using transient DNA transfections in a mammalian cell line. Transfections were carried out by liposome-mediated DNA delivery using lipofectamine (Life Technologies, Inc., Gaithersburg, MD, USA). Experiments were performed according to manufacturer's instructions, varying the number of cells, amount of transfection reagent, and amount of DNA to determine optimum conditions. Typically, 60 x 15mm tissue culture plates containing approximately  $3 \times 10^5$  to  $1 \times 10^6$  cells were employed. After transfections using either pCMV-BL, pCMV-dBL, or pCMV-BLlgM, transfected cells were resuspended in 50 mM Tris-Cl (pH 7.4), 0.1 mM EDTA containing PMSF and leupeptin, swollen on ice for 10 min, then lysed using a Dounce homogenizer. After centrifugation at 800 x g for 6 min, supernatant (cytosolic fraction) was recentrifuged at 30 psi for 20 minutes in a Beckman AirFuge. Pellets from both centrifugations (which include membranes and nuclei) were combined. Each fraction was assayed for activity using the chromogenic substrate PADAC, added to a final concentration of 20 mM (Calbiochem, Corp.). Absorbance at 570 nm was measured using the auto-rate assay of a Kontron Model 9310 spectrophotometer. To assess secreted  $\beta$ -lactamase levels, PADAC assays were carried out on the cell-free media after transfections.  $\beta$ -lactamase enzyme activity was measured using PADAC (-Calbiochem, Corp.) which serves as a chromogenic substrate of  $\beta$ -lactamase activity (Schindler and Huber, Enzyme Inhibitors, Brodbede, Ed., pp 169-176, Verlag Chemie, Weinheim (1980)). A 500  $\mu$ M PADAC stock was made in water, filtered through a 0.22  $\mu$ m filter, and added to media to give a final concentration of 20  $\mu$ M. Decreases in absorbance at 570 nm were measured using the auto-rate assay of a Kontron UV/Vis spectrophotometer.

The data in Figure 5 show that at 48 hours after transfection with lipofectamine, large amounts of  $\beta$ -lactamase are secreted from cells transfected with pCMV-BL. The cellular activity seen with this construct is presumably the enzyme contained in secretory granules in the process of being exported. In contrast the activity seen using pCMV- $\Delta$ BL is completely localized to the cellular fraction. Based on the magnitude of this activity, we estimate that the enzyme from the secretory  $\beta$ -lactamase construct represents 5-10% of total cellular protein made per 24 hours per cell. The activity measured using the membrane construct was found almost exclusively in the membrane fractions.

In order to characterize the polarity of the active membrane form of  $\beta$ -lactamase, whole cell assays were carried out. Transient transfections of human lung adenocarcinoma with pCMV-BLlgM were carried out.  $\beta$ -Lactamase activity was detected only if the assay media was in contact with the cells, indicating that the enzyme must be membrane-bound located on the exterior face of the membrane. Activity was not detected using the same method when a stable cell line expressing the intracellular form of  $\beta$ -lactamase was used as a control, indicating that the substrate does not penetrate cells.

To further confirm the localization of the membrane-form of  $\beta$ -lactamase, stable lines were generated for use in immunohistochemistry experiments. To create stable lines, large-scale transfections in A549 cells were performed. Since pCMV-BL, pCMV- $\Delta$ BL, and pCMV-BLlgM contain the neomycin<sup>R</sup> gene, stable lines could be selected after passaging the lines in media containing the antibiotic, G418. Clonal lines were derived which secrete  $\beta$ -lactamase (pCMV-BL/A549), lines which synthesize an intracellular  $\beta$ -lactamase (pCMV- $\Delta$ BL/A549), and lines which synthesize membrane-bound  $\beta$ -lactamase (pCMV-BLlgM/A549). Cells from each clone were used for immunohistochemistry using a primary rabbit anti- $\beta$ -lactamase antibody followed by a fluorescein-labeled secondary goat anti-rabbit antibody. In this test, cells were not fixed prior to treating with the antibodies. Only the stable line expressing membrane-bound  $\beta$ -lactamase displayed fluorescent labeling above background levels (data not shown).

**(vi)  $\beta$ -Lactamase Delivery to Cells Confers Sensitivity to Cephalosporin Prodrugs**

**A.  $\beta$ -Lactamase efficiently activates 5798W93 and 1614W94**

Prodrugs of methotrexate (5798W93) and 5-fluorouracil (1614W94) represent the parent drugs linked to cephalothin. The kinetic parameters of prodrug activation were measured by incubating various concentrations of prodrug with purified  $\beta$ -lactamase followed by HPLC analysis to determine the rate of prodrug conversion.  $\beta$ -Lactamase efficiently activates both 5798W93 and 1614W94 with a  $k_{cat}/K_M$  (specificity constant) of 272 and 67  $\text{sec}^{-1} \text{mM}^{-1}$ , respectively.

### B. Combination of the $\beta$ -Lactamase Gene with 5798W93 and 1614W94 Confers Toxicity

We have evaluated the *in vitro* toxicity of the  $\beta$ -lactamase prodrugs in the presence and absence of the  $\beta$ -lactamase gene. Cytotoxicity was quantitated by determining  $IC_{50}$ s in treated A549 human lung adenocarcinoma cells using an SRB-based growth inhibition assay (Nair et al., J. Med. Chem., 32, 1277-1279 (1989)).

In the absence of the  $\beta$ -lactamase gene, methotrexate was 10-fold more toxic than the methotrexate prodrug 5798W93, and fluorouracil was 20-fold more toxic than the fluorouracil prodrug 1614W94 (Table 1). When A549 cells which contained stable integrated copie(s) of the secretory  $\beta$ -lactamase gene (A549-BL) were tested, methotrexate and its prodrug 5798W93 were equally toxic (Table 1). This experiment implies that the delivery of the  $\beta$ -lactamase gene to tumor cells will make them sensitive to cephalosporin prodrugs.

The relatively small differential between the toxicity of methotrexate and 5-fluorouracil and their respective prodrugs in the absence of the  $\beta$ -lactamase gene was unexpected. This is because, for both parent drugs, the mechanism of action is well understood and the chemical modification made by attaching cephalothin to these compounds should clearly detoxify the drugs. For example, transport of methotrexate into cells depends on availability of the terminal glutamate moiety which is blocked in 5798W93. Toxicity of 5-fluorouracil depends on the availability of the N1 group since this group is necessary for glycosidic bond formation and concomitant nucleoside formation. The N1 group is blocked in 1614W94. It is clear that the observed toxicity of these prodrugs *in vitro* reflects some degree of chemical instability of the prodrugs which could result in significant breakdown of the prodrugs during the 72-hour incubation utilized in the  $IC_{50}$  determination.

Support for this notion comes from two lines of evidence. The first is that no toxicity is observed when either prodrug is given to mice at a dose equivalent to an  $LD_{100}$  for the parental drug. The lack of toxicity in these cases is explained by the relatively short half life of the drug *in vivo* ( $t_{1/2} \approx 20$  minutes) in contrast to the exposure of cells to the prodrug for 72 hours *in vitro*.

The second line of evidence is shown by direct measurement of *in vitro* toxicity by short-term assays (3 hour exposure of cells to prodrug). Using a sensitive assay for cell toxicity, a 6-[<sup>3</sup>H]-deoxyuridine based assay which measures inhibition of thymidylate synthase and DNA synthesis, we could measure toxicity over time as short as a three hour interval. During this shorter interval, the differential between prodrug and parent drug increased significantly (Table 2). These data are consistent with the idea that the prodrug toxicities reported in Table 1 result from chemical instability of the prodrugs over the long time-course (72 hours) of those experiments.

**(vi) Antitumor Evaluation of Secretory  $\beta$ -Lactamase *in vivo* Using Liposome-Mediated DNA Delivery**

Secretory  $\beta$ -lactamase and cytosine deaminase DNA constructs were compared for antitumour effects in mice bearing subcutaneous (s.c.) A549 human lung adenocarcinoma tumours. Results are shown in Table 3. Plasmid DNA expression vectors encoding either cytosine deaminase (CD) or secretory  $\beta$ -lactamase (BL) under the transcriptional control of the non-specific CMV promoter were encapsidated in cationic liposomes (25 $\mu$ g DNA; 25 nmol liposomes). Mice bearing A549 s.c. tumours were treated with five intratumoral injections of liposomal DNA. Prodrug therapy (1614W94 (50 mg/kg; i.p., qd x 5) or 5-FC (500 mg/kg; i.p., qd x 5) was initiated two days after DNA treatment. Inhibition of tumour growth was determined on day 47. Both CD and BL constructs resulted in similar antitumour activity *in vivo*. 1614W94 administration resulted in about 60% inhibition of tumour growth (Table 3). 5-FC administration resulted in about 70% inhibition of tumour growth, whereas DNA liposomes alone and 5-FU alone (25mg/kg, i.p., qd x 5) resulted in only about 20% inhibition of tumour growth (Table 3). Thus, liposomal DNA/5-FU prodrug combinations resulted in s.c. tumour regressions.

Secretory  $\beta$ -lactamase and cytosine deaminase DNA constructs were also evaluated by intrathoracic (i.t.) injection of liposomal DNA into the pleural space of mice bearing tumors. Results are shown in Table 4. Mice bearing human large cell lung H460 i.t. tumours received DNA encoding either CD or BL under the transcriptional control of the CMV promoter. DNA was dosed by i.t. injection on days 6, 7, 12 and 13. Prodrugs for the respective enzyme were dosed on days 7-16 (5-FC, 500 mg/kg; 1614W94, 70 mg/kg; i.p., qd x 10). Animal survival was evaluated 30 days after tumour implantation. All nontreated mice and mice treated with 5-FU (30 mg/kg i.p.,



qd x 5) died from tumour by 30 days. CMV-BL/1614W94 treatment increased survival to 60%, and CMV-CD/5-FC treatment also increased the survival to 40% (Table 4)

**Table 1 Cytotoxicity (SRB Assay)**

	IC <sub>50</sub>	
	A549	A549-BL
Methotrexate	10 nM	N.D.
1 $\mu$ M, 3h		
5798W93	100 nM	N.D.
1 $\mu$ M, 3h		
5-Fluorouracil	1.9 $\mu$ M	1.4 $\mu$ M
1 $\mu$ M, 5h		
1614W94	40 $\mu$ M	1.7 $\mu$ M
1 $\mu$ M, 5h		

**Table 2 Cytotoxicity (6-[<sup>3</sup>H]-Deoxyuridine Assay)**

	% Inhibition	
	A549	A549-BL
Methotrexate	82 $\pm$ 4	88 $\pm$ 6
1 $\mu$ M, 3h		
5798W93	3 $\pm$ 2	38 $\pm$ 3
1 $\mu$ M, 3h		
5-Fluorouracil	95 $\pm$ 8	91 $\pm$ 10
1 $\mu$ M, 5h		
1614W94	-2 $\pm$ 3	33 $\pm$ 6
1 $\mu$ M, 5h		

Table 3

**Antitumour Effects of Secretory  $\beta$ -Lactamase and Cytosine Deaminase Genes in Mice Bearing Subcutaneous A549 Human Lung Adenocarcinoma**

5

Therapy Group	Tumour Volume (mm <sup>3</sup> )	Percentage Inhibition (Relative to Control)
Phosphate Buffered Saline (No DNA)	1268 $\pm$ 212	0
CMV-BL and 1614W94	511 $\pm$ 86	60
CMV-CD and 5-Fluorocytosine	380 $\pm$ 237	70
5-Fluorouracil alone	1021 $\pm$ 37	19

**Table 4**  
**Antitumour Effects of Secretory  $\beta$ -Lactamase and Cytosine Deaminase Genes in Mice Bearing Intrathoracic H460 Human Large Cell Lung Tumours**

	<b>Therapy Group</b>	<b>Mean Days of Survival</b>	<b>p Value</b>	<b>Increased Life Span (%)</b>
5	Phosphate Buffered Saline (No DNA)	20	-	-
	CMV-CD + PBS	23	0.245	15
	CMV-CD + 5-Fluorocytosine	27	0.009	40
	CMV-BL + 1614W94	32	0.012	60
10	SEQ ID NO 1 = JM30	SEQ ID NO 2 = JM31	SEQ ID NO 3 = JM32	
	SEQ ID NO 4 = CGN3	SEQ ID NO 5 = CGN4	SEQ ID NO 6 = JM1	
	SEQ ID NO 7 = JM2	SEQ ID NO 8 = pCMV-BL		
	SEQ ID NO 9 = JM301	SEQ ID NO 10 = pCMV- $\Delta$ BL		
	SEQ ID NO 11 = MEM1	SEQ ID NO 12 = MEM2		
15	SEQ ID NO 13 = MEM3	SEQ ID NO 14 = pCMV-BLigM		

SEQ ID No 8 PCMV-BL

CGATGT ACGGGCCAGA TATACGCGTT GACATTGATT ATTGACTAGT  
 TATTAATAGT AATCAATTAC GGGGTCATTA GTTCATAGCC CATATATGGA GTTCCGCGTT  
 ACATAACTTA CGGTAAATGG CCCGCCTGGC TGACCGCCCA ACGACCCCGG CCCATTGACG  
 TCAATAATGA CGTATGTTCC CATAGTAACG CCAATAGGGA CTTTCCATTG ACGTCAATGG  
 GTGGACTATT TACGGTAAAC TGCCCACTTG GCAGTACATC AAGTGTATCA TATGCCAAGT  
 ACGCCCCCTA TTGACGTCAA TGACGGTAAA TGGCCCGCCT GGCATTATGC CCAGTACATG  
 ACCTTATGGG ACTTTCTAC TTGGCAGTAC ATCTACGTAT TAGTCATCGC TATTACCATG  
 GTGATGCGGT TTGGCAGTA CATCAATGGG CGTGGATAGC GGTTTGACTC ACGGGGATTT  
 CCAAGTCTCC ACCCCATTGA CGTCAATGGG AGTTTGTITT GGCACCAAAA TCAACGGGAC  
 TTTCCAAAAA GTCGTAACAA CTCGCCCCA TTGACGCAA TGGGCGGTAG GCGGTACGG  
 TGGGAGGTCT ATATAAGCAG AGCTCTCTGG CTAAGTAGAG AACCACTGC TTAAGTGGCT

Hind III (683)

TATCGAAATT AATACGACTC ACTATAGGGA GACCGGAAGC TTGCCACC ATG AGT ATT  
 Met Ser Ile  
 1

CAA CAT TTC CGT GTC GCC CTT ATT CCC TTT TTT GCG GCA TTT TGC CTT  
 Gln His Phe Arg Val Ala Leu Ile Pro Phe Phe Ala Ala Phe Cys Leu  
 5 10 15

CCT GTT TTT GCT CAC CCA GAA ACG CTG GTG AAA GTA AAA GAT GCT GAA  
 pro val phe ala his pro glu thr leu val lys val lys asp ala glu  
 20 25 30 35

GAT CAG TTG GGT GCA CGA GTG GGT TAC ATC GAA CTG GAT CTC AAC AGC  
 Asp Gln Leu Gly Ala Arg Val Gly Tyr Ile Glu Leu Asp Leu Asn Ser  
 40 45 50

Xmr

GGT AAG ATC CTT GAG AGT TTT CGC CCC GAA GAA CGT TTT CCA ATG ATG  
 Gly Lys Ile Leu Glu Ser Phe Arg Pro Glu Glu Arg Phe Pro Met Met  
 55 60 65

AGC ACT TTT AAA GTT CTG CTA TGT GGC GCG GTA TTA TCC CGT ATT GAC  
 Ser Thr Phe Lys Val Leu Leu Cys Gly Ala Val Leu Ser Arg Ile Asp  
 70 75 80

GCC GGG CAA GAG CAA CTC GGT CGC CGC ATA CAC TAT TCT CAG AAT GAC  
Ala Gly Gln Glu Gln Leu Gly Arg Arg Ile His Tyr Ser Gln Asn Asp  
85 90 95

TTG GTT GAG TAC TCA CCA GTC ACA GAA AAG CAT CTT ACG GAT GGC ATG  
Leu Val Glu Tyr Ser Pro Val Thr Glu Lys His Leu Thr Asp Gly Met  
100 105 110 115

ACA GTA AGA GAA TTA TGC AGT GCT GCC ATA ACC ATG AGT GAT AAC ACT  
Thr Val Arg Glu Leu Cys Ser Ala Ala Ile Thr Met Ser Asp Asn Thr  
120 125 130

PvuI (1110)

GCG GCC AAC TTA CTT CTG ACA ACG ATC GGA GGA CCG AAG GAG CTA ACC  
Ala Ala Asn Leu Leu Leu Thr Thr Ile Gly Gly Pro Lys Glu Leu Thr  
135 140 145

GCT TTT TTG CAC AAC ATG GGG GAT CAT GTA ACT CGC CTT GAT CGT TGG  
Ala Phe Leu His Asn Met Gly Asp His Val Thr Arg Leu Asp Arg Trp  
150 155 160

GAA CCG GAG CTG AAT GAA GCC ATA CCA AAC GAC GAG CGT GAC ACC ACG  
Glu Pro Glu Leu Asn Glu Ala Ile Pro Asn Asp Glu Arg Asp Thr Thr  
165 170 175

ATG CCT GTA GCA ATG GCA ACA ACG TTG CGC AAA CTA TTA ACT GGC GAA  
Met Pro Val Ala Met Ala Thr Thr Leu Arg Lys Leu Leu Thr Gly Glu  
180 185 190 195

CTA CTT ACT CTA GCT TCC CGG CAA CAA TTA ATA GAC TGG ATG GAG GCG  
Leu Leu Thr Leu Ala Ser Arg Gln Gln Leu Ile Asp Trp Met Glu Ala  
200 205 210

GAT AAA GTT GCA GGA CCA CTT CTG CGC TGG GCC CTT CCG GCT GGC TGG  
Asp Lys Val Ala Gly Pro Leu Leu Arg Ser Ala Leu Pro Ala Gly Trp  
215 220 225

TTT ATT GCT GAT AAA TCT GGA GCC GGT GAG CGT GGG TCT CGC GGT ATC  
Phe Ile Ala Asp Lys Ser Gly Ala Gly Glu Arg Gly Ser Arg Gly Ile  
230 235 240

ATT GCA GCA CTG GGG CCA GAT GGT AAG CCC TCC CGT ATC GTA GTT ATC  
Ile Ala Ala Leu Gly Pro Asp Gly Lys Pro Ser Arg Ile Val Val Ile  
245 250 255

TAC ACG ACG GGG AGT CAG GCA ACT ATG GAT GAA CGA AAT AGA CAG ATC  
Tyr Thr Thr Gly Ser Gln Ala Thr Met Asp Glu Arg Asn Arg Gln Ile  
260 265 270 275

XbaI(1556) ApaI (1562)

GCT GAG ATA GGT GCC TCA CTG ATT AAG CAT TGG TAATCTAGAG GGCCCTATTC  
Ala Glu Ile Gly Ala Ser Leu Ile Lys His Trp

TATAGTGTCA CCTAAATGCT AGAGCTCGCT GATCAGCCTC GACTGTGCTT TCTAGTTGCC  
 AGCCATCTGT TGTITGCCCC TCCCCCGTGC CTTCCTTGAC CCTGGAAGGT GCCACTCCCA  
 CTGTCCITTC CTAATAAAAT GAGGAAATTG CATCGCATTG TCTGAGTAGG TGTCATTCTA

PvuII

TTCTGGGGGG TGGGGTGGGG CAGGACAGCA AGGGGGAGGA TTGGAAGAC AATAGCAGGC

BamHI (1861)

ATGCTGGGGA TCGGTGGGC TCTATGGAAC CAGCTGGGGC TCGAGGGGGG ATCCCCACGC  
 GCCCTGTAGC GCGGCATTAA GCGCGGCGGG TGTGGTGGTT ACGCGCAGCG TGACCGCTAC  
 ACTTGCCAGC GCCCTAGGCG CCGCTCCTTT CGCTTTCTTC CCTTCCTTC TCGCCACGTT  
 CGCCGGCTTT CCCCCTCAAG CTCTAAATCG GGCATCCCTT TTAGGGTTC GATTAGTGC  
 TTTACGGCAC CTCGACCCCA AAAAACITGA TTAGGGTGAT GGTTCACGTA GTGGGCCATC  
 GCCCTGATAG ACGGTTTTTC GCCTTTACTG AGCACTCTTT AATAGTGGAC TCTGTTCCTA  
 AACTGGAACA ACACTCAACC CTATCTCGGT CTATTCITTT GATTTATAAG ATTTCATCG  
 CCATGTAAAA GTGTTACAAT TAGCAITAAA TTACTTCTTT ATATGCTACT ATTCTTTTGG

EcoRI 2321

CTTCGTTTAC GGGGTGGGTA CCGAGCTCGA ATTCTGTGGA ATGTGTGTCA GTTAGGGTGT  
 GGAAGATCCC CAGGCTCCCC AGGCAGGCAG AAGTATGCAA AGCATGCATC TCAATTAGTC  
 AGCAACACAGG TGTGGAAGT CCCCAGGCTC CCCAGCAGGC AGAAGTATGC AAGCATGCA  
 TCTCAATTAG TCAGCAACCA TAGTCCCGCC CCTAACTCCG CCCATCCCGC CCTAACTCC  
 GCCCAGTTCC GCCCATTTCT CGCCCCATGG CTGACTAATT TTTTITATTT ATTCAGAGGC  
 CGAGGCCGCC TCGGCCTCTG AGCTATTCCA GAAGTAGTGA GGAGGCTTTT TTGGAGGCCT

SmaI (2671)

AGGCTTTTGC AAAAAGCTCC CGGGAGCTTG GATATCCATT TTCGGATCTG ATCAAGAGAC  
 AGGATGAGGA TCGTTTCGCA TGATTGAACA AGATGGATTG CACGCAGGTT CTCGGGCCGC  
 TTGGTGGAG AGGCTATTTC GCTATGACTG GGCACAACAG ACAATCGGCT GCTCTGATGC  
 CGCGGTGTTT CGGCTGTCTG CGCAGGGGCG CCCGGTTCTT TTGTCAAGA CCGACCTGTC

PstI (2909)

PvuII (2964)

CGTGCCCTG AATGAAGTC AGGACGAGGC AGCGGGCTA TCGTGGCTGG CCACGACGGG

CGTTCCTTGC GCAGCTGTGC TCGACGTTGT CACTGAAGCG GGAAGGGACT GGCTGCTATT  
 GGGCGAAGTG CCGGGGCAGG ATCTCCTGTC ATCTCACCTT GCTCCTGCC AGAAAGTATC  
 CATCATGGCT GATGCAATGC GCGGCGTGCA TACGCTTGAT CCGGTACCT GCCCATTGCA  
 CCACCAAGCG AAACATCGCA TCGAGCGAGC ACGTACTCGG ATGGAAGCG GTCTTGTCGA  
 TCAGGATGAT CTGACGAAG AGCATCAGGG GCTCGGCCA GCCGAACGTG TCGCCAGGCT  
 CAAAGGCGCG ATGCCGAGC GCGAGGATCT CGTGTGACC CATGGCGATG CCTGCTTGCC  
 GAATATCATG GTGAAAATG GCCGCTTTTC TGGATTCATC GACTGTGCC GGCTGGGTGT  
 GCGCGACCGC TATCAGGACA TAGCGTTGGC TACCGTGAT ATTGCTGAAG AGCTTGGCGG  
 CGAATGGGCT GACCGCTTCC TCGTGCTTTA CGGTATCGCC GCTCCCGATT CGCAGCGCAT  
 CGCCTTCTAT CGCCTTCTTG ACGAGTTCTT CTGAGCGGGA CTCTGGGGTT CGAAATGACC  
 GACCAAGCGA CGCCCAACT GCCATCAGCA GATTTCGATT CCACCGCGCG CTTCATGAA  
 AGGTTGGGCT TCGGAATCGT TTTCCGGGAC GCGGCTGGA TGATCTCCA GCGCGGGGAT  
 CTCTGCTGG AGTTCTTCGC CCACCCCAAC TTGTTTATTG CAGCTTATAA TGGTTACAA  
 TAAAGCAATA GCATCACAA TTTACAAAT AAAGCATTTT TTTCACTGCA TTCTAGTTGT

BamHI (3830)

GGTTTGTC AACTCATCA TGTATCTTAT CATGTCTGGA TCCCGTCGAC CTCGAGAGCT  
 TGGCGTAATC ATGGTCATAG CTGTTTCTG TGTGAAATG TTATCCGCTC ACAATTCAC  
 ACAACATAG AGCCGGAAGC ATAAAGTGTA AAGCCTGGGG TGCCTAATGA GTGAGCTAAC

PvuII (4029)

TCACATTAAT TGGGTTGCGC TCACTGCCCG CTTTCAGTC GGGAAACCTG TCGTGCCAGC  
 TGCAATTAAT AATCGCCAA CGCGCGGGGA GAGGCGGTTT GCGTATTGGG CGCTCTTCCG  
 CTCTCTCGCT CACTGACTCG CTGCGCTCGG TCGTTCGGCT GCGCGAGCGG GTATCAGTCT  
 ACTCAAAGCG GGTAAACGG TTATCCACAG AATCAGGGA TAACGCAGGA AAGAACATGT  
 GAGCAAAAGG CCAGCAAAAG GCCAGGAACC GTAAAAGGC CGCGTTGCTG GCGTTTTTCC  
 ATAGGCTCCG CCCCCTGAC GAGCATCACA AAATCGAGC CTCAGTCAG AGGTGGCGAA  
 ACCCGACAGG ACTATAAGA TACCAGGCGT TTCCCCTGG AAGCTCCCTC GTGCGCTCTC  
 CTGTTCCGAC CCGCGGCTT ACCGGATACC TGTCGCGCTT TCTCCCTCG GGAAGCGTGG

CGCTTCTCA ATGCTCACGC TGTAGGTATC TCAGTTCGGT GTAGGTGGIT CGCTCCAAGC  
 TGGGCTGTGT GCACGAACCC CCCGTTACGC CGACCGCTG CGCCTTATCC GGTAACATAC  
 GTCTTGAGTC CAACCCGGTA AGACACGACT TATCGCCACT GGCAGCAGCC ACTGGTAACA  
 GGATTAGCAG AGCGAGGTAT GTAGGCGGTG CTACAGAGTT CTGGAAGTGG TGGCCTAACT  
 ACGGCTACAC TAGAAGGACA GTATTTGGTA TCTGCGCTCT GCTGAAGCCA GTTACCTTCG  
 GAAAAAGAT TGTTAGTCTT TGATCCGGCA AACAAACAC CGCTGGTAGC GGTGGTTTTT  
 TTGTTTGCAA GCAGCAGATT ACGCGCAGAA AAAAAGGATC TCAAGAAGAT CCTTGTATCT  
 TTCTACGGG GTCTGACGCT CAGTGGAAACG AAAACTCACG TTAAGGGATT TTGGTCATGA  
 GATTATCAA AAGGATCTTC ACCTAGATCC TTTTAAATTA AAAATGAAGT TTTAATCAA  
 TCTAAAGTAT ATATGAGTAA ACTTGGTCTG ACAGTTACCA ATGCTTAATC AGTAGGCAC  
 CTATCTCAGC GATCTGTCTA TTCTGTTCAT CCATAGTTGC CTGACTCCCC GTCTGTGAGA  
 TAACTACGAT ACGGGAGGGC TTACCATCTG GCCCAGTAGC TGCAATGATA CCGCGAGACC  
 CACGCTCACC GGCTCCAGAT TTATCAGCAA TAAACCAGCC AGCCGGAAGC GCGGACGCCA  
 GAAGTGGTCC TGCAACTTTA TCCGCTTCCA TCCAGTCTAT TAATTGTTGC CGGGAAGCTA  
 GAGTAAGTAG TTCGCCAGTT AATAGTTTGC GCAACGTTGT TGCCATTGCT ACAGGCATCG  
 TGGTGTCACG CTCGTCGTTT GGTATGGCTT CATTGAGCTC CGGTTCCCAA CGATCAAGGC

PvuI (5467)

GAGTTACATG ATCCCCATG TTGTGCAAAA AAGCGGTTAG CTCCTTCGGT CCTCCGATCG  
 TTGTGAGAAG TAAGTTGGCC GCAGTGTAT CACTCATGGT TATGGCAGCA CTGCATAATT

ScaI (5578)

CTCTTACTGT CATGCCATCC GTAAGATGCT TTTCTGTGAC TGGTGAGTAC TCAACCAAGT  
 CATTCTGAGA ATAGTGTATG CGCGACCGA GTTGCTCTTG CCCGCGCTGA ATACGGGATA

XmnI (5695)

ATACCGCGCC ACATAGCAGA ACTTTAAAG TGCTCATCAT TGGAAAACGT TCITCGGGGC  
 GAAAACTCTC AAGGATCTTA CCGCTGTGTA GATCCAGTTC GATGTAACCC ACTCGTGAC  
 CCAACTGATC TTCAGCATCT TTTACTTTCA CCAGCGTTTC TGGGTAGCA AAAACAGGAA  
 GGCAAAATGC CGCAAAAAAG GGAATAAGGG CGACACGGAA ATGTTGAATA CTCATACTCT



TCCTTTTCA ATATTATTGA AGCATTATC AGGGTTATTG TCTCATGAGC GGATACATAT  
TTGAATGTAT TTAGAAAAAT AACCAAATAG GGGTTCCGGG CACATTTCCT CGAAAAGTGC  
CACCTGACGT CGACGGATCG GGAGATCTCC CGATCCCCTA TGGTCGACTC TCAGTACAAT  
CTGCTCTGAT GCCGCATAGT TAAGCCAGTA TCTGCTCCCT GCTTGTTGTG TGGAGGTCGC  
TGAGTAGTGC GCGAGCAAAA TTTAAGCTAC AACAAAGGCAA GGCTTGACCG ACAATTGCAT  
Nrul (6229)  
GAAGAATCTG CTTAGGGTTA GCGGTTTTGC GCTGCTTCG

SEQ ID NO 10 - PCMV-ΔBL

GACGGATCGG GAGATCTCCC GATCCCTAT GGTGACTCT CAGTACAATC TGCTCTGATG 60  
 CCGCATAGTT AAGCCAGTAT CTGCTCCCTG CTGTGTGTT GGAGGTGCT GAGTAGTGCG 120  
 CGAGCAAAT TTAAGCTACA ACAAGGCAAG GCTTGACCGA CAATTGCATG AAGAATCTGC 180  
 NruI (206)  
 TTAGGGTTAG GCGTTTTCG CTGCTTCGCG ATGTACGGGC CAGATATACG CGTTGACATT 240  
 SpeI (249)  
 GATTATTGAC TAGTTATTAA TAGTAATCAA TTACGGGGTC ATTAGTTCAT AGCCCCATATA 300  
 TGGAGTTCG CGTTACATAA CTTACGTAA ATGGCCCGCC TGGCTGACCG CCCAACGACC 360  
 CCGGCCCATI GACGTCAATA ATGACGTATG TTCCCATAGT AACGCCAATA GGGACTTTCC 420  
 ATTGACGTCA ATGGGTGGAC TATTTACGGT AAACGTCCCA CTGGCAGTA CATCAAGTGT 480  
 NdeI (483)  
 ATCATATGCC AAGTACGCCC CCTATTGACG TCAATGACGG TAAATGGCCC GCCTGGCATT 540  
 SnaBI (588)  
 ATGCCAGTA CATGACCTTA TGGGACTTTC CTACTTGGCA GTACATCTAC GTATTAGTCA 600  
 TCGCTATTAC CATGGTGATG CGGTTTTCG AGTACATCAA TGGGCGTGGG TAGCGGTTTG 660  
 ACTCACGGGG ATTTCCAAGT CTCACCCCA TTGACGTCAA TGGGAGTTTG TTTTGCACC 720  
 AAAATCAACG GGACTTTCCA AAATGTCGTA ACAACTCCG CCCATTGACG CAATGGGGG 780  
 GTAGGCGTGT ACGGTGGGAG GTCTATATAA GCAGAGCTCT CTGGCTAACT AGAGAACCCA 840  
 Hind III (891)  
 CTGCTTAACT GGCTTATCGA AATTAAATACG ACTCACTATA GGGAGACCGG AAGCTTGCCA 900  
 CC ATG CAC CCA GAA ACG CTG GTG AAA GTA AAA GAT GCT GAA GAT CAG 947  
 Met His Pro Glu Thr Leu Val Lys Val Lys Asp Ala Glu Asp Gln  
 1 5 10 15  
 TTG GGT GCA CGA GTG GGT TAC ATC GAA CTG GAT CTC AAC AGC GGT AAG 995  
 Leu Gly Ala Arg Val Gly Tyr Ile Glu Leu Asp Leu Asn Ser Gly Lys  
 20 25 30  
 XmnI (1020)  
 ATC CTT GAG AGT TTT CGC CCC GAA GAA CGT TTT CCA ATG ATG AGC ACT 1043  
 Ile Leu Glu Ser Phe Arg Pro Glu Glu Arg Phe Pro Met Met Ser Thr  
 35 40 45

TTT AAA GTT CTG CTA TGT GGC GCG GTA TTA TCC CGT ATT GAC GCC GGG Phe Lys Val Leu Leu Cys Gly Ala Val Leu Ser Arg Ile Asp Ala Gly 50 55 60	1091
CAA GAG CAA CTC GGT CGC CGC ATA CAC TAT TCT CAG AAT GAC TTG GTT Gln Glu Gln Leu Gly Arg Arg Ile His Tyr Ser Gln Asn Asp Leu Val 65 70 75	1139
ScaI (1141) GAG TAC TCA CCA GTC ACA GAA AAG CAT CTT ACG GAT GGC ATG ACA GTA Glu Tyr Ser Pro Val Thr Glu Lys His Leu Thr Asp Gly Met Thr Val 80 85 90 95	1187
AGA GAA TTA TGC AGT GCT GCC ATA ACC ATG AGT GAT AAC ACT GCG GCC Arg Glu Leu Cys Ser Ala Ala Ile Thr Met Ser Asp Asn Thr Ala Ala 100 105 110	1235
PvuI (1252) AAC TTA CTT CTG ACA ACG ATC GGA GGA CCG AAG GAG CTA ACC GCT TTT Asn Leu Leu Leu Thr Thr Ile Gly Gly Pro Lys Glu Leu Thr Ala Phe 115 120 125	1283
TTG CAC AAC ATG GGG GAT CAT GTA ACT CGC CTT GAT CGT TGG GAA CCG Leu His Asn Met Gly Asp His Val Thr Arg Leu Asp Arg Trp Glu Pro 130 135 140	1331
GAG CTG AAT GAA GCC ATA CCA AAC GAC GAG CGT GAC ACC ACG ATG CCT Glu Leu Asn Glu Ala Ile Pro Asn Asp Glu Arg Asp Thr Thr Met Pro 145 150 155	1379
GTA GCA ATG GCA ACA ACG TTG CGC AAA CTA TTA ACT GGC GAA CTA CTT Val Ala Met Ala Thr Thr Leu Arg Lys Leu Leu Thr Gly Glu Leu Leu 160 165 170 175	1427
ACT CTA GCT TCC CGG CAA CAA TTA ATA GAC TGG ATG GAG GCG GAT AAA Thr Leu Ala Ser Arg Gln Gln Leu Ile Asp Trp Met Glu Ala Asp Lys 180 185 190	1475
GTT GCA GGA CCA CTT CTG CGC TCG GCC CTT CCG GCT GGC TGG TTT ATT Val Ala Gly Pro Leu Leu Arg Ser Ala Leu Pro Ala Gly Trp Phe Ile 195 200 205	1523
GCT GAT AAA TCT GGA GCC GGT GAG CGT GGG TCT CGC GGT ATC ATT GCA Ala Asp Lys Ser Gly Ala Gly Glu Arg Gly Ser Arg Gly Ile Ile Ala 210 215 220	1571
GCA CTG GGG CCA GAT GGT AAG CCC TCC CGT ATC GTA GTT ATC TAC ACG Ala Leu Gly Pro Asp Gly Lys Pro Ser Arg Ile Val Val Ile Tyr Thr 225 230 235	1619
ACG GGG AGT CAG GCA ACT ATG GAT GAA CGA AAT AGA CAG ATC GCT GAG Thr Gly Ser Gln Ala Thr Met Asp Glu Arg Asn Arg Gln Ile Ala Glu	1667

240	245	250	255
XbaI (1698) ApaI (1704)			
ATA GGT GCC TCA CTG ATT AAG CAT TGG TAA TCTAGAGGGC CCTATTCTAT			1717
Ile Gly Ala Ser Leu Ile Lys His Trp *			
260		265	
AGTGTCACTT AAATGCTAGA GCTCGCTGAT CAGCCTCGAC TGTGCCTTCT AGTTGCCAGC			1777
CATCTGTGTG TTGCCCCCTC CCCGTGCCTT CCTTGACCCT GGAAGGTGCC ACTCCCACTG			1837
TCCTTTCTTA ATAAATGAG GAAATTGCAT CGCATTGTCT GAGTAGGTGT CATTCTATTC			1897
TGGGGGGTGG GGTGGGGCAG GACAGCAAGG GGGAGGATTG GGAAGACAAT AGCAGGCATG			1957
PvuII (1985) Bam HI (2003)			
CTGGGGATGC GGTGGGCTCT ATGGAACCAG CTGGGGCTCG AGGGGGGATC CCCACGCGCC			2017
CTGTAGCGGC GCATTAAAGC CGGCGGGTGT GGTGGTTACG CGCAGCGTGA CCGCTACACT			2077
TGCCAGCGCC CTAGCGCCCG CTCCTTTCGC TTCTTCCCT TCCTTTCTCG CCACGTTTCGC			2137
CGGCTTTCCC CGTCAAGCTC TAAATCGGGG CATCCCTTTA GGGTCCCGAT TTAGTGCTTT			2197
ACGGCACCTC GACCCCAAAA AACTTGATTA GGGTGATGGT TCACGTAGTG GGCCATCGCC			2257
CTGATAGACG GTTTTTCGCC TTTACTGAGC ACTCTTTAAT AGTGGACTCT TGTTCCAAAC			2317
TGGAACAACA CTCAACCTTA TCTCGTCTA TTCCTTTGAT TTATAAGATT TCCATCGCCA			2377
TGTAAAAGTG TTACAATTAG CATTAATAITTA CTTCCTTATA TGCTACTATT CTTTGGCTT			2437
EcoRI (2463)			
CGTTCACGGG GTGGGTACCG AGCTCGAATT CTGTGGAATG TGTGTCAGTT AGGGTGTGGA			2497
AAGTCCCCAG GCTCCCCAGG CAGGCAGAAG TATGCAAAGC ATGCATCTCA ATTAGTCAGC			2557
AACCAAGTGT GGAAGATCCC CAGGCTCCCC AGCAGGCAGA AGTATGCAAA GCATGCATCT			2617
CAATTAGTCA GCAACCATAG TCCCGCCCCC AACTCCGCCC ATCCCGCCCC TAACTCCGCC			2677
CAGTTCGGCC CATTCTCCGC CCCATGGCTG ACTAATTTTT TTTATTTATG CAGAGGCCGA			2737
StuI (2789)			
GGCCGCTCG GCCTCTGAGC TATTCAGAA GTAGTGAGGA GGCTTTTTTG GAGGCTTAGG			2797
SmaI (2813) EcoRV (2825)			
CTTTTGCAAA AAGCTCCCGG GAGCTTGGAT ATCCATTTC GGATCTGATC AAGAGACAGG			2857
ATGAGGATCG TTTGCGATGA TTGAACAAGA TGGATTGCAC GCAGGTTCTC CGGCGCGTTG			2917
GGTGGAGAGG CTATTCCGCT ATGACTGGGC ACAACAGACA ATCGGCTGCT CTGATGCCGC			2977

CGTGTCCGG CTGTACGCG AGGGGCGCCC GGTCTTTTT GTCAAGACCG ACCTGTCCGG	3037
PstI (3051)	
TGCCCTGAAT GAACTGCAGG ACGAGGCAGC GCGGCTATCG TGGCTGGCCA CGACGGGCGT	3097
PvuII (3106)	
TCCTTGCGCA GCTGTGCTCG ACGTTGTCAC TGAAGCGGGA AGGGACTGGC TGCTATTGGG	3157
CGAAGTGCCG GGGCAGGATC TCCTGTATC TCACCTTGCT CCTGCCGAGA AAGTATCCAT	3217
CATGGTGAT GCAATGCGGC GGCTGCATAC GCTTGATCCG GCTACCTGCC CATTGACCA	3277
CCAAGCGAAA CATCGCATCG AGCGAGCAGC TACTCGGATG GAAGCCGGTC TTGTCGATCA	3337
GGATGATCTG GACGAAGAGC ATCAGGGGCT CGCGCCAGCC GAACTGTTGC CCAGGCTCAA	3397
GGCGCGCATG CCCGACGGCG AGGATCTCGT CGTGACCCAT GCGCATGCCT GCTTGCCGAA	3457
TATCATGGTG GAAAATGGCC GCTTTTCTGG ATTTCATCGAC TGTGCCCGGC TGGGTGTGGC	3517
GGACCGCTAT CAGGACATAG CGTTGGCTAC CGTGATATT GCTGAAGAGC TTGGCGGCGA	3577
ATGGGCTGAC CGCTTCTCTG TGCTTTACGG TATCGCCGCT CCCGATTGCG AGCGCATCGC	3637
CTTCTATCGC CTTCTTGACG AGTTCTTCTG AGCGGGACTC TGGGGTTCGA AATGACCGAC	3697
CAAGCGACGC CCAACCTGCC ATCAGGAGAT TTCGATTCCA CGCGCGCCTT CTATGAAAGG	3757
TGGGCTTCG GAATCGTTTT CCGGAGCGCC GGCTGGATGA TCCTCCAGCG CGGGGATCTC	3817
ATGCTGGAGT TCTTCGCCCA CCCCAACTTG TTTATTGCAG CTTATAATGS TTACAAATAA	3877
BamHI (3972)	
AGCAATAGCA TCACAAATTT CACAAATAA GCATTTTTT CACTGCATTC TAGTTGTGGT	3937
TGTCCAAAC TCATCAATGT ATCTTATCAT GTCTGGATCC CGTGACCTC GAGAGCTTGG	3997
CGTAATCATG GTCATAGCTG TTTCTGTGT GAAATTGTTA TCCGCTCACA ATTCACACA	4057
ACATACGAGC CGGAAGCATA AAGTGTAAG CCTGGGGTGC CTAATGAGTG AGCTAACTCA	4117
PvuII	
CATTAATTGC GTTGCGCTCA CTGCCGCTT TCCAGTCGGG AAACCTGTGC TGCCAGCTGC	4177
ATTAATGAAT CGGCCAACGC GCGGGGAGAG GCGGTTTGGC TATTGGGCGC TCTTCCGCTT	4237
CCTCGCTCAC TGACTCGCTG CGCTCGGTG TTCGGTGC GCGAGCGGTA TCAGCTCACT	4297
CAAAGGCGGT AATACGGTTA TCCACAGAA CAGGGGATAA CGCAGGAAAG AACATGTGAG	4357
CAAAGGCCA GCAAAGGCC AGGAACCGTA AAAAGGCCGC GTTGCTGGCG TTTTCCATA	4417

GGCTCGCCCC	CCCTGACGAG	CATCACAAAA	ATCGACGCTC	AAGTCAGAGG	TGGCGAAACC	4477
CGACAGGACT	ATAAGATAC	CAGGCGTTTC	CCCCTGGAAG	CTCCCTCGTG	CGCTCTCCTG	4537
TTCCGACCCT	GCCGCTTACC	GGATACCTGT	CCGCCTTTCT	CCCTTCGGGA	AGCGTGGCGC	4597
TTTCTCAATG	CTCACGCTGT	AGGTATCTCA	GTTGCGGTGA	GGTCGTTTCG	TCCAAGCTGG	4657
GCTGTGTGCA	CGAACCCCC	GTTCAGCCCC	ACCGCTGCGC	CTTATCCGGT	AACTATCGTC	4717
TTGAGTCCAA	CCCGTAAGA	CACGACTTAT	CGCCTCTGGC	AGCAGCCACT	GGTAACAGGA	4777
TTAGCAGAGC	GAGGTATGTA	GGCGGTGCTA	CAGAGTTCTT	GAAGTGGTGG	CCTAACTACG	4837
GCTACACTAG	AAGGACAGTA	TTTGGTATCT	CGGCTCTGCT	GAAGCCAGTT	ACCTTCGGAA	4897
AAAGAGTTGG	TAGCTCTTGA	TCCGGCAAAC	AAACCACCGC	TGGTAGCGGT	GGTTTTTTTG	4957
TTTGCAAGCA	GCAGATTACG	CGCAGAAAAA	AAGGATCTCA	AGAAGATCCT	TTGATCTTTT	5017
CTACGGGGTG	TGACGCTCAG	TGGAACGAAA	ACTCACGTGA	AGGGATTTTG	GTCATGAGAT	5077
TATCAAAAAG	GATCTTCACC	TAGATCCTTT	TAAATTAATA	ATGAAGTTTT	AAATCAATCT	5137
AAAGTATATA	TGAGTAAACT	TGGTCTGACA	GTTACCAATG	CTTAATCAGT	GAGGCACCTA	5197
TCTCAGCGAT	CTGTCTATTT	CGTTTCATCCA	TAGTTGCCTG	ACTCCCCGTC	GTGTAGATAA	5257
CTACGATACG	GGAGGGCTTA	CCATCTGGCC	CCAGTGCTGC	AATGATACCG	CGAGACCCAC	5317
GCTCACCGGC	TCCAGATTTA	TCAGCAATAA	ACCAGCCAGC	CGGAAGGGCC	GAGCGCAGAA	5377
GTGGTCTGTC	AACTTTATCC	GCCTCCATCC	AGTCTATTAA	TTGTTGCCGG	GAAGCTAGAG	5437
TAAGTAGTTC	GCCAGTTAAT	AGTTTGCGCA	ACGTTGTTGC	CATTGCTACA	GGCATCGTGG	5497
TGTCACGCTC	GTCGTTTGTT	ATGGCTTCAT	TCAGCTCCGG	TTCCCAACGA	TCAAGGCGAG	5557
PvuI (5609)						
TTACATGATC	CCCCATGTTG	TGCAAAAAAG	CGGTTAGCTC	CTTCGGTCCT	CCGATCGTTG	5617
TCAGAAGTAA	GTTGGCCGCA	GTGTTATCAC	TCATGGTTAT	GGCAGCACTG	CATAATTCTC	5677
ScaI (5720)						
TTACTGTCAT	GCCATCCGTA	AGATGCTTTT	CTGTGACTGG	TGAGTACTCA	ACCAAGTCAT	5737
TCTGAGAATA	GTGTATGCGG	CGACCGAGTT	GCTCTTGCCC	GGCGTCAATA	CGGGATAATA	5797
XmnI (5837)						
CCGCGCCACA	TAGCAGAACT	TTAAAAGTGC	TCATCATTGG	AAAACGTTCT	TCGGGGCGAA	5857

AACTCTCAAG GATCTTACCG CTGTTGAGAT CCAGTTGGAT GTAAACCCACT CGTGCACCCA 5917  
ACTGATCTTC AGCATCTTTT ACTTTCACCA GCGTTTCTGG GTGAGCAAAA ACAGGAAGGC 5977  
AAAATGCCGC AAAAAAGGGA ATAAGGGCGA CACGGAAATG TTGAANTACTC ATACTCTTCC 6037  
TTTTTCAATA TTATTGAAGC ATTTATCAGG GTTATTGTCT CATGAGCGGA TAC

SEQ ID No 14 - pCMV - BLigm

GACGGATCGG GAGATCTCCC GATCCCCCTAT GGTGACTCT CAGTACAATC TGCTCTGATG	60
CCGCATAGTT AAGCCAGTAT CTGCTCCCTG CTGTGTGTT GGAGGTCGCT GAGTAGTGCG	120
CGAGCAAAT TTAAGCTACA ACAAGGCAAG GCTTGACCGA CAATTGCATG AAGAATCTGC	180
NruI (206)	
TTAGGGTTAG GCGTTTTCG CTGCTTCGCG ATGTACGGGC CAGATATACG CGTTGACATT	240
SpeI (249)	
GATTATTGAC TAGTTATTAA TAGTAATCAA TTACGGGGTC ATTAGTTCAT AGCCCATATA	300
TGGAGTCCG CGTTACATAA CTTACGGTAA ATGGCCCGCC TGGCTGACCG CCCAACGACC	360
CCCGCCCAT TACGTCAATA ATGACGTATG TTCCCATAGT AACGCCAATA GGGACTTTCC	420
ATTGACGTCA ATGGGTGGAC TATTTACGGT AAACGTGCCA CTTGGCAGTA CATCAAGTGT	480
NdeI (483)	
ATCATATGCC AAGTACGCC CCTATTGACG TCAATGACGG TAAATGGCCC GCCTGGCATT	540
SnaBI (588)	
ATGCCCAGTA CATGACCTTA TGGGACTTTC CTACTTGGCA GTACATCTAC GTATTAGTCA	600
TCGCTATTAC CATGGTGATG CGGTTTTCG AGTACATCAA TGGGCGTGGA TAGCGGTTTG	660
ACTCACGGGG ATTTCCAAGT CTCACCCCA TTGACGTCAA TGGGAGTTTG TTTTGGCACC	720
AAATCAACG GGACTTTCCA AAATGTCGTA ACAACTCCGC CCCATTGACG CAAATGGGCG	780
GTAGGCGTGT ACGGTGGGAG GTCTATATAA GCAGAGCTCT CTGGCTAACT AGAGAACCCA	840
Hind III (891)	
CTGCTTAACT GGCTTATCGA AATTANTACG ACTCACTATA GGGAGACCGG AAGCTTGCCA	900
CC ATG AGT ATT CAA CAT TTC CGT GTC GCC CTT ATT CCC TTT TTT GCG	947
Met Ser Ile Gln His Phe Arg Val Ala Leu Ile Pro Phe Phe Ala	
1 5 10 15	
GCA TTT TGC CTT CCT GTT TTT GCT CAC CCA GAA ACG CTG GTG AAA GTA	995
Ala Phe Cys Leu Pro Val Phe Ala His Pro Glu Thr Leu Val Lys Val	
20 25 30	
AAA GAT GCT GAA GAT CAG TTG GGT GCA CGA GTG GGT TAC ATC GAA CTG	1043
Lys Asp Ala Glu Asp Gln Leu Gly Ala Arg Val Gly Tyr Ile Glu Leu	
35 40 45	
XmnI (1086)	
GAT CTC AAC AGC GGT AAG ATC CTT GAG AGT TTT CGC CCC GAA GAA CGT	1091



Asp	Leu	Asn	Ser	Gly	Lys	Ile	Leu	Glu	Ser	Phe	Arg	Pro	Glu	Glu	Arg		
	50						55					60					
TTT	CCA	ATG	ATG	AGC	ACT	TTT	AAA	GTT	CTG	CTA	TGT	GGC	GCG	GTA	TTA	1139	
Phe	Pro	Met	Met	Ser	Thr	Phe	Lys	Val	Leu	Leu	Cys	Gly	Ala	Val	Leu		
	65						70				75						
TCC	CGT	ATT	GAC	GCC	GGG	CAA	GAG	CAA	CTC	GGT	CGC	CGC	ATA	CAC	TAT	1187	
Ser	Arg	Ile	Asp	Ala	Gly	Gln	Glu	Gln	Leu	Gly	Arg	Arg	Ile	His	Tyr		
	80				85					90					95		
ScaI (1207)																	
TCT	CAG	AAT	GAC	TTG	GTT	GAG	TAC	TCA	CCA	GTC	ACA	GAA	AAG	CAT	CTT	1235	
Ser	Gln	Asn	Asp	Leu	Val	Glu	Tyr	Ser	Pro	Val	Thr	Glu	Lys	His	Leu		
			100						105					110			
ACG	GAT	GGC	ATG	ACA	GTA	AGA	GAA	TTA	TGC	AGT	GCT	GCC	ATA	ACC	ATG	1283	
Thr	Asp	Gly	Met	Thr	Val	Arg	Glu	Leu	Cys	Ser	Ala	Ala	Ile	Thr	Met		
			115					120					125				
PvuI (1318)																	
AGT	GAT	AAC	ACT	GCG	GCC	AAC	TTA	CTT	CTG	ACA	ACG	ATC	GGA	GGA	CCG	1331	
Ser	Asp	Asn	Thr	Ala	Ala	Asn	Leu	Leu	Thr	Thr	Ile	Gly	Gly	Pro			
		130					135					140					
AAG	GAG	CTA	ACC	GCT	TTT	TTG	CAC	AAC	ATG	GGG	GAT	CAT	GTA	ACT	CGC	1379	
Lys	Glu	Leu	Thr	Ala	Phe	Leu	His	Asn	Met	Gly	Asp	His	Val	Thr	Arg		
		145					150				155						
CTT	GAT	CGT	TGG	GAA	CCG	GAG	CTG	AAT	GAA	GCC	ATA	CCA	AAC	GAC	GAG	1427	
Leu	Asp	Arg	Trp	Glu	Pro	Glu	Leu	Asn	Glu	Ala	Ile	Pro	Asn	Asp	Glu		
	160				165				170					175			
CGT	GAC	ACC	ACG	ATG	CCT	GTA	GCA	ATG	GCA	ACA	ACG	TTG	CGC	AAA	CTA	1475	
Arg	Asp	Thr	Thr	Met	Pro	Val	Ala	Met	Ala	Thr	Thr	Leu	Arg	Lys	Leu		
			180					185						190			
TTA	ACT	GGC	GAA	CTA	CTT	ACT	CTA	GCT	TCC	CGG	CAA	CAA	TTA	ATA	GAC	1523	
Leu	Thr	Gly	Glu	Leu	Leu	Thr	Leu	Ala	Ser	Arg	Gln	Gln	Leu	Ile	Asp		
		195						200					205				
TGG	ATG	GAG	GCG	GAT	AAA	GTT	GCA	GGA	CCA	CTT	CTG	CGC	TCG	GCC	CTT	1571	
Trp	Met	Glu	Ala	Asp	Lys	Val	Ala	Gly	Pro	Leu	Leu	Arg	Ser	Ala	Leu		
		210					215					220					
CCG	GCT	GGC	TGG	TTT	ATT	GCT	GAT	AAA	TCT	GGA	GCC	GGT	GAG	CGT	GGG	1619	
Pro	Ala	Gly	Trp	Phe	Ile	Ala	Asp	Lys	Ser	Gly	Ala	Gly	Glu	Arg	Gly		
		225					230					235					
TCT	CGC	GGT	ATC	ATT	GCA	GCA	CTG	GGG	CCA	GAT	GGT	AAG	CCC	TCC	CGT	1667	
Ser	Arg	Gly	Ile	Ile	Ala	Ala	Leu	Gly	Pro	Asp	Gly	Lys	Pro	Ser	Arg		
		240				245					250				255		

ATC GTA GTT ATC TAC ACC ACG GGG AGT CAG GCA ACT ATG GAT GAA CGA Ile Val Val Ile Tyr Thr Thr Gly Ser Gln Ala Thr Met Asp Glu Arg 260 265 270	1715
XbaI (17611)	
AAT AGA CAG ATC GCT CAG ATA GGT GCC TCA CTG ATT AAG CAT TGG TCT Asn Arg Gln Ile Ala Glu Ile Gly Ala Ser Leu Ile Lys His Trp Ser 275 280 285	1763
AGA GAG GGG GAG GTG AGC GCC GAC GAG GAG GGC TTT GAG AAC CTG TGG Arg Glu Gly Glu Val Ser Ala Asp Glu Glu Gly Phe Glu Asn Leu Trp 290 295 300	1811
GCC ACC GCC TCC ACC TTC ATC GTC CTC TTC CTC CTG AGC CTC TTC TAC Ala Thr Ala Ser Thr Phe Ile Val Leu Phe Leu Ser Leu Phe Tyr 305 310 315	1859
AGT ACC ACC GTC ACC TTG TTC AAG GTA GCA CGG CTG TGG CAC AGG GAG Ser Thr Thr Val Thr Leu Phe Lys 320 325	1907
GAG GGT GCA GGG CGA GTG TGG GGC CCA GGG AGC AGC CTG GGC TGG ACG	1955
TCT AGC CCG GAG GCC CCC ACA CCA CCC CAC TGG GTC ATC TCT GCC CCG	2003
GCT CCC TTC CCG ACC ACA GGA AAG CAT TTC ACA CTG TCT CTG TTG CCT	2051
ApaI (2094)	
GTA GGT GAA ATG ATC CCA ACA GAA GAA CAT CGG AGA CCA GAG G	2094
GGCCCTATTC TATACTGTCA CCTAAATGCT AGAGCTCGCT GATCAGCCTC GACTGTGCCT	2154
CTAGTTGCC AGCCATCTGT TGTTCGCCCC TCCCCGTGC CTTCCTTGAC CCTGGAAGGT	2214
GCCACTCCCA CTGTCTTTTC CTAATAAAAT GAGGAAATTG CATCGCATTG TCTGAGTAGG	2274
TGTCATTCTA TTCTGGGGGG TGGGGTGGGG CAGGACAGCA AGGGGGAGGA TTGGAAGAC	2334
PvuII (2375) BamHI (2393)	
AATAGCAGGC ATGCTGGGGA TCGGGTGGGC TCTATGGAAC CAGCTGGGGC TCGAGGGGGG	2394
ATCCCCACGC GCCCTTAGC GCGCATTA GCGCGCGGGG TGTGGTGOTT ACGCGCAGCG	2454

TGACCGCTAC ACTTGCCAGC GCCCTAGCGC CCGCTCCTTT CGCTTCTTC CCTTCCTTC	2514
TCGCCACGTT CGCCGGCTTT CCCCGTCAAG CTCTAAATCG GGGCATCCCT TTAGGGTTCC	2574
GATTTAGTGC TTACGCGCAC CTCGACCCCA AAAAAGTTGA TTAGGGTGAT GGTTCACGTA	2634
GTGGGCCATC GCGCTGATAG ACGGTTTTTC GCCTTTACTG AGCACTCTTT AATAGTGGAC	2694
TCTTGTTCCA AACTGGAACA ACACTCAACC CTATCTCGGT CTATCTTTT GATTTATAAG	2754
ATTTCCATCG CCATGTAAAA GTGTTACAAT TAGCATTAAA TTACTTCTTT ATATGCTACT	2814
EcoRI (2853)	
ATTCTTTTGG CTTCGTTTAC GGGGTGGGTA CCGAGCTCGA ATTCTGTGGA ATGTGTGTCA	2874
GTTAGGGTGT GGAAAGTCCC CAGGCTCCCC AGGCAGGCAG AAGTATGCAA AGCATGCATC	2934
TCAATTAGTC AGCAACCAGG TGTGAAAAGT CCCCAGGCTC CCCAGCAGGC AGAAGTATGC	2994
AAAGCATGCA TCTCAATTAG TCAGCAACCA TAGTCCCGCC CCTAACTCCG CCCATCCCGC	3054
CCCTAACTCC GCCCAGTTCC GCCCATTTCT CGCCCCATGG CTGACTAATT TTTTTATT	3114
StuI (3179)	
ATGCAGAGGC CGAGGCCGCC TCGGCCTCTG AGCTATTCCA GAAGTAGTGA GGAGGCTTTT	3174
SmaI (3203) EcoRV	
TTGGAGGCCT AGGCTTTTGC AAAAAGCTCC CGGGAGCTTG GATATCCATT TTCGGATCTG	3234
ATCAAGAGAC AGGATGAGGA TCGTTTCGCA TGATTGAACA AGATGGATTG CACGCAGGTT	3294
CTCCGGCCGC TTGGGTGGAG AGGCTATTCT GCTATGACTG GGCACAACAG ACAATCGGCT	3354
GCTCTGATGC CGCCGTGTTT CCGCTGTCTG CGCAGGGGCG CCCGGTTCTT TTGTCAAGA	3414
PstI (3441)	
CCGACCTGTC CGGTGCCCTG AATGAAGTGC AGGACGAGGC AGCGCGGCTA TCGTGGCTGG	3474
PvuII (3496)	
CCACGACGGG CGTTCCTTGC GCAGCTGTGC TCGACGTTGT CACTGAAGCG GGAAGGGACT	3534
GGCTGCTATT GGGCGAAGTG CCGGGGCAGG ATCTCTGTGC ATCTCACCTT GCTCCTGCCG	3594
AGAAAGTATC CATCATGGCT GATGCAATGC GCGGCTGCA TACGCTTGAT CCGGCTACCT	3654
GCCCATTCGA CCACCAAGCG AAACATCGCA TCGAGCGAGC ACGTACTCGG ATGGAAGCCG	3714
GTCTTGTCGA TCAGGATGAT CTGGACGAAG AGCATCAGGG GCTCGGCCA GCCGAAGTGT	3774
TCGCCAGGCT CAAGGCGCGC ATGCCGACG GCGAGGATCT CGTCGTGACC CATGGCGATG	3834
CCTGCTTGCC GAATATCATG GTGGAAGATG GCCGTTTTT TGGATTATC GACTGTGGCC	3894

GGCTGGGTGT GCGGACCGC TATCAGGACA TAGCGTTGGC TACCCGTGAT ATTGCTGAAG	3954
AGCTTGCGCG CGAATGGGCT GACCGCTTCC TCGTGCTTTA CGGTATCGCC GCTCCCGATT	4014
CGCAGCGCAT CGCCTTCTAT CGCCTTCTTG ACGAGTTCTT CTGAGCGGGA CTCTGGGGTT	4074
CGAAATGACC GACCAAGCGA CGCCCAACCT GCCATCAGCA GATTTCGATT CCACCGCGCG	4134
CTTCTATGAA AGGTGGGGCT TCGGAATCGT TTTCGGGAC GCCGGCTGGA TGATCCTCCA	4194
GCGCGGGGAT CTCATGCTGG AGTTCCTCGC CCACCCCAAC TTGTTTATTG CAGCTTATAA	4254
TGGTTACAAA TAAAGCAATA GCATCACAAA TTTCACAAAT AAAGCATTTT TTCACTGCA	4314
BamHI (4362)	
TTCTAGTTGT GGTTTGTCCA AACTCATCAA TGTATCTTAT CATGTCTGGA TCCCGTCGAC	4374
CTCGAGAGCT TGGCGTAATC ATGGTCATAG CTGTTTCCTG TGTGAAATTG TTATCCGCTC	4434
ACAAATCCAC ACAACATACG AGCCGGAAGC ATAAAGTGTA AAGCCTGGGG TGCCTAATGA	4494
GTGAGCTAAC TCACATTAAT TGCCTTGC GC TCACTGCCCG CTTCCAGTC GGGAAACCTG	4554
PvuII (4561)	
TCGTGCCAGC TGCATTAATG AATCGGCCAA CGCGCGGGA GAGGCGGTT GCGTATTGGG	4614
CGCTCTTCCG CTTCTCGCT CACTGACTCG CTGCGCTCGG TCGTTCGGCT GCGGCGAGCG	4674
GTATCAGCTC ACTCAAAGGC GGTAAACGG TTATCCACAG AATCAGGGGA TAACGCAGGA	4734
AAGAACATGT GAGCAAAAGG CCAGCAAAAG GCCAGGAACC GTAAAAAGGC GCGTGTGCTG	4794
GCGTTTTTCC ATAGGCTCCG CCCCCCTGAC GAGCATCACA AAAATCGAGC CTCAGTCAG	4854
AGGTGGCGAA ACCCGACAGG ACTATAAAGA TACCAGGCGT TTCCCCCTGG AAGCTCCCTC	4914
GTGCGCTCTC CTGTTCCGAC CCTGCCGCTT ACOGGATACC TGTCCGCCTT TCTCCCTTGG	4974
GGAAGCGTGG CGCTTTCTCA ATGCTCACGC TGTAGGTATC TCAGTTCGGT GTAGGTCGTT	5034
CGCTCCAAGC TGGGCTGTGT GCACGAACCC CCCGTTACGC CCGACCGCTG CGCCTTATCC	5094
GGTAACTATC GTCTTGAGTC CAACCCGGTA AGACACGACT TATCGCCACT GGCAGCAGCC	5154
ACTGGTAACA GGATTAGCAG AGCGAGGTAT GTAGGCGGTG CTCAGAGATT CTTGAAGTGG	5214
TGGCCTAACT ACGGCTACAG TAGAAGGACA GTATTGGTA TCTGCGCTCT GCTGAAGCCA	5274
GTTACCTTCG GAAAAAGAGT TGGTAGCTCT TGATCCGGCA AACAAACCA CGCTGGTAGC	5334
GGTGGTTTTT TTGTTTGCAA GCAGCAGATT ACGCGCAGAA AAAAAGGATC TCAAGAAGAT	5394

CCTTTGATCT TTTCTACGGG GTCTGACGCT CAGTGGAAACG AAAACTCAGC TTAAGGGATT	5454
TTGGTCATGA GATTATCAAA AAGGATCTTC ACCTAGATCC TTTTAAATTA AAAATGAAGT	5514
TTTAAATCAA TCTAAAGTAT ATATGAGTAA ACTTGGTCTG ACAGTTACCA ATGCTTAATC	5574
AGTGAGGCAC CTATCTCAGC GATCTGTCTA TTTGTTTCAT CCATAGTTGC CTGACTCCCC	5634
GTGCTGTAGA TAACTACGAT ACGGGAGGGC TTACCATCTG GCCCCAGTGC TGCAATGATA	5694
CGCGAGACC CACGCTCACC GGCTCCAGAT TTATCAGCAA TAAACCAGCC AGCCGGAAGG	5754
GCCGAGCGCA GAAGTGTCC TGCAACTTTA TCCGCCTCCA TCCAGTCTAT TAATTGTTGC	5814
CGGGAAGCTA GAGTAAGTAG TTCGCCAGTT AATAGTTTGC GCAACGTTGT TGCCATTGCT	5874
ACAGGCATCG TGGTGTACG CTCGTCGTTT GGTATGGCTT CATTGAGTCT CGGTTCCCAA	5934
CGATCAAGGC GAGTTACATG ATCCCCCATG TTGTGCAAAA AAGCGGTTAG CTCCTTCGGT	5994
CCTCCGATCG TTGTCAGAAG TAAGTTGGCC GCACTGTTAT CACTCATGGT TATGGCAGCA	6054
	ScaI (6110)
CTGCATAATT CTCTTACTGT CATGCCATCC GTAAGATGCT TTTCTGTGAC TGGTGAGTAC	6114
	XmnI (6227)
TCAACCAAGT CATCTGAGA ATAGTGATG CGGCGACCGA GTTGCTCTTG CCCGGCGTCA	6174
ATACGGGATA ATACCGCGCC ACATAGCAGA ACTTTAAAG TGCTCATCAT TGGAAAACGT	6234
TCTTCGGGCG GAAAACTCTC AAGGATCTTA CCGCTGTTGA GATCCAGTTC GATGTAACCC	6294
ACTCGTGCAC CCAACTGATC TTCAGCATCT TTTACTTTCA CCAGCGTTTC TGGGTGAGCA	6354
AAAACAGGAA GGCAAAATGC CGCAAAAAAG GGAATAAGGG CGACACGGAA ATGTTGAATA	6414
CTCATACTCT TCCTTTTTC ATATTATTGA AGCATTATC AGGGTTATTG TCTCATGAGC	6474
GGATACATAT TTGAATGTAT TTAGAAAAAT AAACAAATAG GGGTTCCGCG CACATTTCCT	6534
CGAAAAGTGC CACCTGACGT C	6555

**CLAIMS:**

1. Use of a molecular chimaera for the manufacture of a medicament for use with a prodrug in the therapy of lung cancer, the molecular chimaera comprising a transcriptional regulatory DNA sequence derived from a gene encoding a lung-associated protein or a neuroendocrine marker protein and, operatively linked to the transcriptional regulatory DNA sequence, a DNA sequence encoding a heterologous enzyme capable of catalysing the conversion of the prodrug into an agent toxic to a lung cancer cell.

2. Use according to claim 1 wherein the transcriptional regulatory DNA sequence is derived from the genes coding for proopiomelanocortin (POMC), chromogranin A, gastrin releasing peptide, uteroglobin or surfactant protein B.

3. Use according to claim 1 or 2 wherein the heterologous enzyme is  $\beta$ -lactamase, cytosine deaminase, thymidine kinase, penicillin amidase, alkaline phosphatase,  $\beta$ -glucuronidase or carboxypeptidase.

4. Use according to any of claims 1 to 3 wherein the transcriptional regulatory DNA sequence comprises a promoter.

5. Use according to claim 4 wherein the transcriptional regulatory DNA sequence also comprises an enhancer.

6. Use according to any of claims 1 to 5 wherein the prodrug is a phenylacetylated derivative of a toxic agent or a cephalosporin conjugate of a toxic agent.

7. Use according to claim 6 wherein the prodrug is a phenyl acetylated derivative of adriamycin, methotrexate or 2-aminopurine or a cephalosporin conjugate of 5-fluorouracil, methotrexate or adriamycin.

8. A molecular chimaera for use in therapy of lung cancer with a prodrug, the molecular chimaera comprising a transcriptional regulatory DNA sequence derived from a gene encoding a lung-associated protein or a neuroendocrine marker protein and, operatively linked to the transcriptional regulatory DNA sequence, DNA sequence

encoding a heterologous enzyme capable of catalysing the conversion of the prodrug into an agent toxic to a lung cancer cell.

- 5 9. A molecular chimaera according to claim 8 wherein the transcriptional regulatory DNA sequence is derived from the genes coding for proopiomelanocortin (POMC), chromogranin A, gastrin releasing peptide, uteroglobin or surfactant protein B.
- 10 10. A molecular chimaera according to claim 8 or 9 wherein the heterologous enzyme is  $\beta$ -lactamase, cytosine deaminase, thymidine kinase, penicillin amidase, alkaline phosphatase,  $\beta$ -glucuronidase or carboxypeptidase.
- 15 11. A molecular chimaera according to any of claims 8 to 10 wherein the transcriptional regulatory DNA sequence comprises a promoter.
12. A molecular chimaera according to claim 11 wherein the transcriptional regulatory DNA sequence also comprises an enhancer.
- 20 13. A vector containing a molecular chimaera according to any of claims 8 to 12.
14. A vector according to claim 13 which is a viral vector.
15. A vector according to claim 14 which is a retroviral vector.
- 25 16. A packaging cell line containing a viral vector according to claim 14 or 15.
17. An infective virion generated from a packaging cell line according to claim 16.
18. An infective virion according to claim 17 which is a retrovirus.
- 30 19. A liposome containing a molecular chimaera according to any of claims 8 to 12 or a vector according to any of claims 13 to 15.
20. Use of a vector according to any of claims 13 to 15, a packaging cell line according to claim 16, an infective virion according to claim 17 or 18 or a liposome
- 35

according to claim 19 for the manufacture of a medicament for use with a prodrug in the therapy of lung cancer.

21. Use according to any of claim 20 wherein the prodrug is a phenylacetylated derivative of a toxic agent or a cephalosporin conjugate of a toxic agent.

22. Use according to claim 21 wherein the prodrug is a phenyl acetylated derivative of adriamycin, methotrexate or 2-aminopurine or a cephalosporin conjugate of 5-fluorouracil, methotrexate or adriamycin.

23. A pharmaceutical formulation suitable for use in the therapy of lung cancer with a prodrug, the formulation comprising a molecular chimera according to any of claims 8 to 12, a vector according to any of claims 13 to 15, a packaging cell line according to claim 16, an infective virion according to claim 17 or 18 or a liposome according to claim 19 together with a pharmaceutically acceptable carrier

24. Use according to any of claims 1 to 7 or 20 to 22 wherein the therapy comprises administration of the medicament by calcium phosphate transfection, electroporation, microinjection, liposomal transfer, ballistic barrage of retroviral infection.

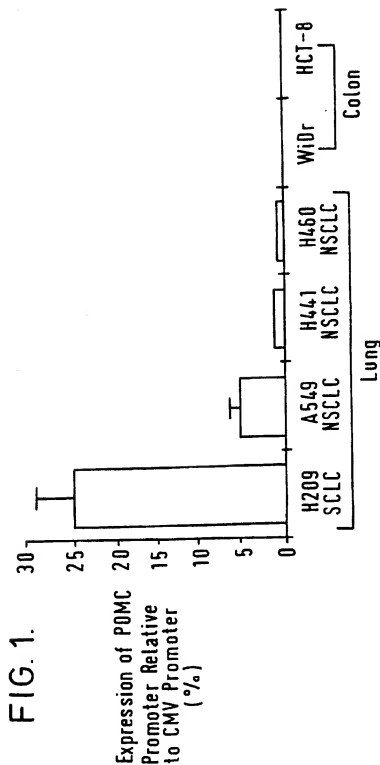
25. Use according to claim 24 wherein the therapy comprises administration of the medicament by liposomal transfer.

26. Use according to claim 25 wherein the therapy comprises administration of the medicament by liposomal transfer using a bronchoscope.

27. Method of treating cancer which comprises administering to a mammal an effective amount of chimera as claimed in any one of claims 8 to 12, a vector as claimed in any one of claims 13 to 15, a packaging cell line as claimed in claim 16, an infective virion as claimed in claim 17 or 18 or a liposome according to claim 19.



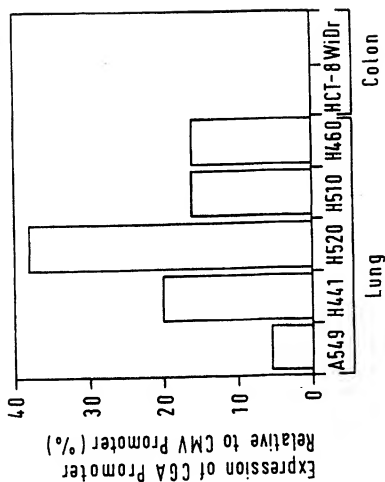
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TARGETING SMALL CELL LUNG TUMORS  
WITH POMC PROMOTERS

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SPECIFICITY OF CGA: A LUNG TUMOR SPECIFIC  
PROMOTER

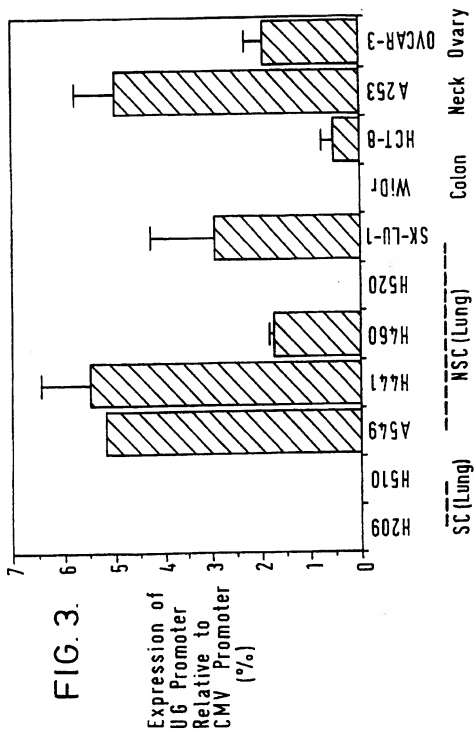
FIG. 2.



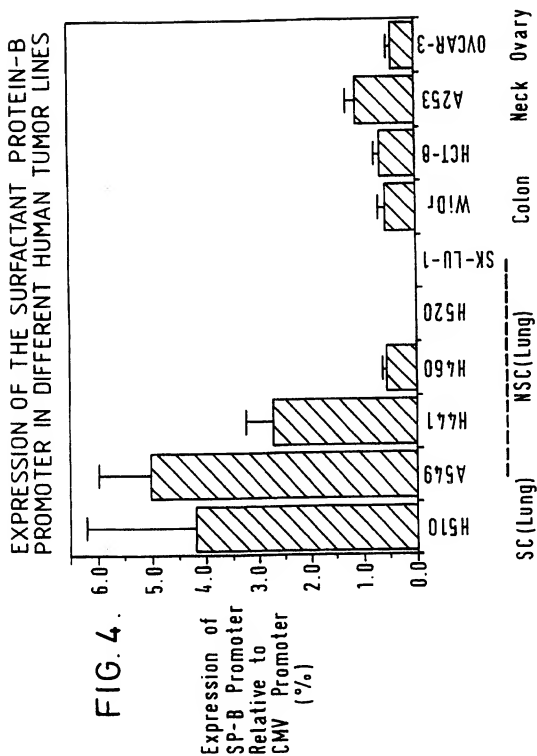
CGA = CHROMOGRANIN A

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# EXPRESSION OF THE UTEROGLOBIN PROMOTER IN DIFFERENT HUMAN TUMOR LINES



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